Pathoadaptation of the Intracellular Bacteria *Shigella* and *Chlamydia*: Virulence, Antivirulence, and Tissue Tropism

by

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DEDICATION

This work is dedicated to my parents, Scott and Laura Bliven, for their love, encouragement, and support during this venture.

"They say a little knowledge is a dangerous thing,

but it's not one half so bad as a lot of ignorance."

Sir Terry Pratchett (1948-2015) (430)

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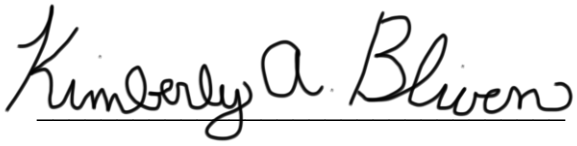

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ABSTRACT

Pathoadaptation of the Intracellular Bacteria *Shigella* and *Chlamydia*: Virulence, Antivirulence, and Tissue Tropism

Kimberly A. Bliven, Doctor of Philosophy 2015

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Horizontal transfer of pathogenicity islands or plasmids can enable bacterial adaptation to novel pathogenic niches. Success in the new niche is dependent on conservation of advantageous genes and concomitant inactivation or loss of deleterious or unnecessary alleles. For example, the acquisition of type three secretion systems (T3SSs) by *Shigella* and *Chlamydia* species facilitated entry into and exploitation of eukaryotic host cells. Conversely, both microbes experienced significant pseudogenization and genome reduction as a result of adaptation to the intracellular lifestyle. The pathogenic *Shigella* evolved from commensal *Escherichia coli* ancestors following acquisition of a large virulence plasmid carrying the T3SS. Antivirulence genes (AVGs) inhibitory to the new intracellular niche have been inactivated or lost from all strains. To identify novel *Shigella* AVGs, a contracted version of the large virulence plasmid was created using the minimal genes required for T3SS expression and assembly. Termed the *Shigella* minimally invasive plasmid (*S*mip), this construct was transformed into a *S. flexneri*

strain cured of the large virulence plasmid (BS103), and the virulence phenotypes of the resulting strain characterized. BS103/*S*mip was capable of host cell entry, demonstrating the presence of a functional T3SS. However, although expression of the *S*mip in commensal *E. coli* isolates permitted host cell entry, invasion was severely attenuated compared to wild type *Shigella*. In two *E. coli*/*S*mip isolates, T3SS expression was severely inhibited at the level of the T3SS transcriptional regulators, *virF* and *virB*, indicating that that multiple virulence inhibitors (potential AVGs) are likely present in these strains. As part of a second project exploring pathogen evolution, the activity and expression of the arginine decarboxylase enzyme (AaxB) in *Chlamydia* was examined. *Chlamydia* species infect a wide variety of different hosts and tissues, although the genetic basis for host and tissue tropism is still under investigation. While functional AaxB is conserved in the majority of zoonotic *Chlamydia* species, two distinct mutational events have inactivated this protein in serovars of the human-specific *C. trachomatis* strains. Furthermore, uncleaved protein production peaked at 20 hours post-infection, while cleavage occurred optimally at 44 hours. Potential roles for the AaxB enzyme in virulence and tissue tropism will be discussed.

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LIST OF ABBREVIATIONS

ArgDC	Arginine decarboxylase
AVG	Antivirulence gene
EB	Elementary body
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
HGT	Horizontal gene transfer
HUS	Hemolytic uremic syndrome
IS	Insertion element
M cell	Microfold cell
MDR	Multidrug-resistant
PAI	Pathogenicity island
pINV	Large virulence plasmid of *Shigella*/EIEC
PMN	Polymorphonuclear leukocyte
PvlArgDC	Pyruvoyl-dependent ArgDC
RB	Reticulate body
SER	*Shigella* entry region
***S*mip**	*Shigella* minimally invasive plasmid
STEC	Shiga toxin-producing *E. coli*
Stx	Shiga toxin
T3SS	Type three secretion system

CHAPTER 1: INTRODUCTION

Selective pressures imposed on bacteria by their environment facilitate the emergence and evolution of pathogens. These include, but are not limited to: the immune system of the potential host(s); other bacteria, which may produce toxic compounds such as colicins; predators, such as amoeba; environmental stressors such as pH, temperature, and osmotic pressure; and the availability of vital nutrients. To overcome these challenges, microbes must adapt or risk host- or bacterial-mediated killing, predation, or starvation. Bacteria with mutations that confer a selective advantage within a particular environment survive to replicate and pass these new genes on to their progeny; conversely, bacteria with less advantageous genotypes are outcompeted and eventually lost from the population. This is a very simplistic model, however, and not always strictly applicable to actual events. Additional factors such as genetic drift and antagonistic pleiotropy also shape the genomes of microbial communities (111; 584).

The evolution of a bacterium towards a more pathogenic phenotype occurs through gain- or loss-of-function mechanisms resulting from two basic types of selection: positive selection, the selection for advantageous mutations or genes, and negative (purifying) selection, the removal of deleterious mutations or genes. An additional category, neutral selection, includes genes that confer neither an advantage nor disadvantage to the evolving pathogen. Eventually, many genes under neutral selection are eradicated from the bacterial genome through random inactivation and/or deletion events. In highly specialized, niche-adapted bacteria, such as *Chlamydia*, *Buchnera*, and *Mycobacterium leprae*, neutral selection has resulted in severe genome reduction (108; 334; 497; 513).

Gain-of-function events involve either the beneficial mutation of existing genes to modify protein function, or the acquisition of entirely new genes from outside the bacterial genome. Advantageous gain-of-function mutations are selected for and vertically passed to the pathogen's resulting progeny. Genes can also be spread laterally by horizontal gene transfer (HGT), which includes the conjugation, transduction, or transformation of genetic information between strains or species. The transfer of mobile genetic elements, such as plasmids, bacteriophages, and integrons, accelerates pathogen evolution by rapidly dispersing a favorable gene(s) across a microbial community. The swift spread of antibiotic resistance genes via HGT, for example, has become a major health concern, and plasmids or integrons harboring multiple resistance genes are now commonly found in many prevalent pathogens (49).

Loss-of-function events involve the inactivation or deletion of existing genes. Loss-of-function mutations are more frequent than gain-of-function mutations, partially due to the greater probability of interrupting protein expression or function via errors accumulated during bacterial replication, and partially due to disruptive mechanisms such as insertion elements (253). Such events constitute an important feature of pathogen evolution that can rapidly drive pathogens into specific, novel niches. In certain instances, loss-of-function mutations can actually promote pathogen fitness by increasing virulence (62).

In this thesis, the pathogenic evolution of two Gram-negative, intracellular bacterial pathogens, *Shigella* and *Chlamydia*, will be examined. *Shigella* evolved directly from ancestral commensal *Escherichia coli* strains following the acquisition of a large virulence plasmid which harbors a type three secretion system (T3SS) required for host

cell invasion and spread (487). To integrate this new system into the bacterial genome, antivirulence genes (AVGs) incompatible with the new intracellular lifestyle were inactivated or deleted to optimize pathogenesis (62; 339). The search for novel *Shigella* AVGs was pursued by introducing the *Shigella* minimally invasive plasmid (*S*mip), a contracted version of the large virulence plasmid, into commensal *E. coli* strains and studying various virulence phenotypes of the resulting strains. Next, the activity and expression of the arginine decarboxylase (ArgDC) enzyme in *Chlamydia* will be discussed (60). We speculate that the presence of functional ArgDC in the zoonotic *Chlamydia* species is the result of positive selection to promote survival in macrophages, while ArgDC inactivation events in the human-specific *C. trachomatis* serovars are indicative of neutral selection.

First, a brief overview on pathogen evolution will be presented, followed by separate literature reviews on *Shigella* virulence and antivirulence factors. Next, the characterization and utilization of the *S*mip in *Shigella* will demonstrate the potential practical applications of this tool (64). In the following chapter, the *S*mip is introduced into commensal *E. coli* strains as a prospective method to identify novel antivirulence genes (AVGs) in *Shigella*. A literature review on *Chlamydia* virulence and evolution will introduce our second pathogen. Finally, the functionality and expression of the ArgDC enzyme, AaxB, in various *Chlamydia* species will be presented and proposed as a determinant of tissue tropism. An extended discussion and future directions for these projects will conclude the thesis.

CHAPTER 2: EVOLUTION OF BACTERIAL PATHOGENS WITHIN THE HUMAN HOST

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Note: The table and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

Selective pressures within the human host, including interactions with innate and adaptive immune responses, exposure to medical interventions such as antibiotics, and competition with commensal microbes all facilitate the evolution of bacterial pathogens. In this chapter, we present examples of pathogen strategies that emerged as a result of selective pressures within the human host niche, and discuss the resulting co-evolutionary 'arms race' between these organisms. In bacterial pathogens, many of the genes responsible for these strategies are encoded on mobile pathogenicity islands (PAIs) or plasmids, underscoring the importance of horizontal gene transfer (HGT) in the emergence of virulent microbial species.

INTRODUCTION

The success or failure of a pathogen is entirely dependent on its ability to survive, reproduce, and spread to a new host or environment. Host immune systems, predators, microbial competitors, parasites, and environmental resource limitations all exert selective pressures that shape the genomes of microbial populations (542). Host evolutionary fitness, meanwhile, is reliant on its capability to survive and reproduce; the host must effectively curtail diseases that weaken either of these abilities.

Dawkins *et al.* (1979) suggest that the conflicting drives between host and pathogen have led to an evolutionary arms race, where an asymmetric 'attack-defense' strategy has come into play (120). At the basic level, this concept suggests that when a host evolves new defenses to thwart a pathogen's attack, the pathogen is forced to adapt a more impressive attack strategy to penetrate the heightened defenses. In response, the host must once again develop new defenses to cope with the new attack mechanism, and

the cycle continues. Evolutionarily fit pathogens, which are able to survive, replicate, and spread effectively within the host, have an improved chance of passing their genes on to the next generation. Similarly, host genotypes are more likely to persist within the population if those particular individuals are more capable of controlling or resisting infection. Evolution, therefore, is driven by positive directional selection in the 'arms race' model; eventually, beneficial alleles should become fixed in a population. Another model favors frequency-dependent (balancing) selection, a process that maintains rare alleles and therefore preserves polymorphic diversity within a population (586). Simply put, allele fixation is prevented because different bacterial alleles confer distinct advantages to the pathogen in the presence of different host alleles. Evidence exists within nature for both directional and frequency-dependent selection, and both types probably occur in bacterial populations.

In this chapter, we explore the host-pathogen interface and offer examples of pathogen adaptation in response to common host selective pressures (Table 1). Although we will focus our attention exclusively on bacterial pathogens within the human host, many of the concepts discussed in this review are readily applicable to other organisms, such as viruses, parasites, and fungi, which can infect a wide range of hosts including plants, animals, and amoeba (110; 356; 533).

As a final note, much of the evidence presented here to support presumed evolutionary events is either speculation from what is currently known or suspected about host and microbial biology, or the result of artificial laboratory-induced evolution during serial passaging of bacterial strains. Due to the sheer enormity of evolutionary timescales, defining the precise origins of and factors driving natural evolutionary events is often a

Table 1. Examples of pathogenic mechanisms to evade or overcome selective pressures within the human host.

Selective Pressures	**Pathogenic mechanisms to evade or overcome these pressures**
Physical barriers in host (i.e., mucosal epithelium)	Mucinases Enterotoxins Exfoliative toxins Transcytosis through M cells
Host complement	Complement inhibitor protein C3 protease
Sequestration of host resources (e.g. iron)	Enterobactin/aerobactin systems
Host B- and T-cell lymphocytes	Cytotoxins T3SS-mediated apoptosis
Antibiotics, antimicrobial peptides	Efflux pumps Mutations in antimicrobial targets Enzymes to inactivate antibiotics (e.g. beta-lactamases)
Bacterial colicins	Colicin immunity proteins
Bacterial T6SSs	T6S immunity proteins

difficult undertaking.

ANTAGONISTIC PLEIOTROPY AND THE FITNESS COST/BENEFIT ANALYSIS

At the most basic level, the theory of natural selection stipulates that, within a bacterial population, beneficial traits will be conserved (selected for), and deleterious traits eventually discarded (selected against). The actual evolutionary process is considerably more complex, however, due to the existence of genetic drift (the change in genetic diversity of a population due to random chance) and antagonistic pleiotropy.

Antagonistic pleiotropy is the concept that a single gene may control more than one phenotype, some of which may be beneficial to the organism, and some deleterious (584). Therefore, a gene may confer a selective advantage within one particular environment, but its expression could be detrimental within a different environment. Conservation of this gene ultimately is determined by the overall necessity of the gene to the organism's fitness. Bacterial pathogens may evolve mechanisms to neutralize the deleterious effects arising from antagonistic pleiotropy, while at the same time conserving the beneficial ones. Temporal regulation is a powerful tool to ensure that specific genes are only turned on when required, and turned off to prevent detrimental expression within a particular environment. Certain outer membrane proteins or systems are temporally regulated within the host, as they may provide a marker for recognition by the host immune system. Flagella expression, for example, is down-regulated by *Salmonella enterica* serovar Typhi *in vivo* to avoid activation of the host inflammatory response; however, outside the host, motility is likely important for the bacterium to seek out and scavenge nutrients from the environment (468). Other bacteria avoid the deleterious effects of a gene through gene inactivation; mutants that lose functionality of

the gene once it becomes deleterious can out-compete the wild type parent strain, and eventually these mutants will dominate the population. *Pseudomonas aeruginosa*, an opportunistic pathogen of cystic fibrosis patients, often switches to a mucoid phenotype *in vivo* as a result of overproduction of the exopolysaccharide alginate, which allows for the production of a bacterial biofilm in the lung (73; 591). MucA is a *P. aeruginosa* transmembrane protein that binds to and represses the sigma factor AlgU, which acts as the transcriptional activator of the alginate synthesis operon. AlgU activates AlgR, a suppressor of T3SS expression; when *mucA* is expressed, therefore, so are the T3SS genes. During acute infection, the T3SS plays an essential role in establishment of the bacterium within the respiratory tract. Once infection has been established, however, chronic infection appears to favor loss of T3SS and a switch to biofilm production (233). Both of these phenotypes are at least partially driven by various mutations in *mucA* that lead to derepression of AlgU, subsequent production of alginate, and suppression of the T3SS (591). Hauser (2009) speculates that loss of the T3SS protects the bacterium from eventual recognition by the host, as patients infected with *P. aeruginosa* develop antibodies against T3SS effector proteins; conversely, biofilm production likely allows for the persistence of the organism in the respiratory tract (233). Finally, certain bacteria simply tolerate deleterious fitness costs if the benefits of expressing the gene outweigh the negative effects. Antibiotic resistance mutations that allow bacteria to survive exposure to antimicrobials often come with a significant fitness disadvantage, for example, and secondary compensatory mutations in these strains may eventually arise to restore fitness rather than lose resistance (492).

THE IMPACT OF HOST-PATHOGEN INTERACTIONS ON MICROBIAL EVOLUTION

Inside the host, a successful pathogen will pilfer resources to survive, replicate, and eventually escape; concomitantly, the host will attempt to recognize and subsequently rid the body of the intruder. Co-evolution between host and pathogen naturally occurs as a result of these interactions (369). For practical purposes, we will restrict our discussion to bacterial adaptation within the human host, but it is important to recognize that many of these concepts are applicable to pathogens of other hosts as well, such as plants or amoeba (290; 310; 431). As novel genetic variants within the human population emerge that prove more successful at preventing or overcoming infection, only pathogen variants that can surmount or avoid this new response will be successful. Natural host defenses, which take much longer to evolve than their microbial counter-parts, have also been supplemented by antibiotics and modern medical interventions over the last century. Such man-made developments place added pressures on microbes to adapt (118). Host innate and adaptive immune responses and modern medical interventions are all selective pressures that contribute to pathogen evolution within the human host. Furthermore, microbial competition, either against other pathogens or commensal microbes, also shapes pathogen genomes.

Bacteria have several advantages over the human host when it comes to evolution: first, their generation times are significantly shorter, leading to a much more rapid selection of beneficial alleles within a population. In conjunction with a shorter generation time, bacterial populations are typically larger, which may allow for greater genetic diversity from which to select. Lastly, many bacteria utilize horizontal gene transfer (HGT), which accounts for the rapid spread of advantageous alleles between strains or even species (583). Virulence genes are commonly located on transferred

pathogenicity islands (PAIs), segments of the genome associated with mobility elements, such as integrase genes or transposons, and a G+C content that differs from the remainder of the genome (254).

Host selective pressures: The innate and adaptive immune systems

The innate immune system is one of the first challenges encountered by the incoming pathogen following host contact. These diverse host defenses include physical barriers such as the mucosal epithelium, activation of the complement cascade, circulating antimicrobial peptides and cytokines, leukocytes, and sequestration of host nutrients away from pathogenic bacteria. In addition to evading innate immune mechanisms, the bacteria must also prevent or avoid adaptive immune responses such as B cell antibody production and, in the case of intracellular bacteria, T cell-mediated cytotoxicity. Pathogenic bacteria have evolved different approaches to overcome these host defenses.

In the human colon alone, intestinal microbiota concentrations average 10^{11} microorganisms per gram gut content, while 3×10^{8} prokaryotes are thought to colonize the entire skin surface of the human adult (582). Consequently, bacteria that exploit more hostile and less frequently occupied niches may gain a selective edge in survival by avoiding sites of high competition. Natural structural barriers, however, typically prevent pathogens from engaging deeper host tissues. Physical blocks to infection include the intestinal and respiratory mucosa, the blood-brain barrier, the blood-CSF (cerebral spinal fluid) barrier, and the placental barrier (135). Most of these structures consist of a single layer of epithelial or endothelial cells bound closely together by tight junctions, adherens junctions, and desmosomes, resulting in the construction of an effective barrier to

preclude bacteria from passively crossing (135; 552). Gastric and respiratory epithelia support an additional protective coating of mucus, which consists primarily of mucin glycoproteins and antimicrobial molecules (350). Mucin glycoproteins, produced by epithelial goblet cells and submucosal glands, can either remain cell-associated or undergo secretion into the mucosa, where they contribute to the viscous layer of mucus that can effectively trap microbes (321). Additionally, non-specific antimicrobials, such as defensins and lysozymes, and antigen-specific antimicrobials, such as IgG and secretory IgA, also limit the growth of microbes within the mucosa (350). Bacterial pathogens have developed numerous mechanisms to counteract these defenses.

The mucosal barrier can be broken down by mucinases such as the Pic enzyme of *Shigella* and Enteroaggregative *Escherichia coli* (EAEC) (217; 239). The *pic* gene is located on a chromosomal pathogenicity island in *Shigella*, and bounded upstream and downstream by insertion (IS)-like elements in EAEC, indicating a history of horizontal gene transfer in these pathogens (239). This potential gene transfer is intriguing as mucin degradation is also important for certain gastrointestinal commensals, which metabolize mucin glycoproteins for energy (507). It is tempting to speculate that these enzymes first evolved within human commensal bacteria as a means of nutrient acquisition, and only later spread to emerging pathogens to confer passage through the mucosal surface. Such a concept would support the hypothesis proposed by Rasko *et al.*, who suggest that commensal *E. coli* act as 'genetic sinks' for pathogenic *E. coli* isolates (444). Other pathogens, such as *Yersinia enterocolitica*, avoid the thickest layers of the mucosal layer by targeting microfold (M) cells within the small intestine for uptake (275; 350). These specialized epithelial cells sample microorganisms residing in the intestinal lumen and

present them to immune cells in the underlying lymphoid tissue. M cells are situated in the region of the epithelium known as the dome, which lacks mucin-secreting goblet cells (350).

Next, to breach the epithelial/endothelial barrier, pathogens must either actively cross using microbial-mediated processes, or opportunistically cross following disruption of barrier integrity. Some pathogens, such as *Bacteroides fragilis* and *Staphylococcus aureus*, directly break cell-cell junctions (227; 590). *B. fragilis*, an opportunistic pathogen, encodes a zinc-dependent metalloprotease toxin, BFT (*B. fragilis* enterotoxin), which cleaves the extracellular domain of E-cadherin, a host zonula adherens protein (590). Like the *pic* genes of *Shigella* and EAEC, the *bft* gene is carried on a pathogenicity island present in all enterotoxigenic *Bacteroides fragilis* strains (182). *S. aureus* induces bullous impetigo and staphylococcal scalded skin syndrome through the actions of three exfoliative toxins (ETs): ETA, ETB, and ETD (227). The ETs act as serine proteases which cleave human desmoglein 1, a transmembrane protein of desmosomes. The genes encoding these toxins are carried on different mobile genetic elements: the ETA gene is carried by a family of Sa1int phages; the ETB gene is plasmid-encoded; and the ETD gene localizes to a 9 kB pathogenicity island (268; 596). Other pathogens, such as *Shigella*, *Salmonella*, and *Listeria*, transcytose through M cells in the gut to gain access to the basolateral surface of the intestinal epithelium (272). Because these specialized host cells overlay Peyer's Patches (or gut-associated lymphoid tissue (GALT)), enteric bacteria transcytosed through M cells must then contend with macrophages, T lymphocytes, B lymphocytes, and dendritic cells.

As a putative example of counter-evolution, the human host may have developed mechanisms to avoid bacterial-mediated adhesion processes. *Helicobacter pylori* binds to the adhesion decoy Muc1, a mucin expressed on the surface of epithelial cells in the gastrointestinal tract (349). Muc1 is subsequently shed from the epithelial surface along with coupled bacteria, precluding long-term adhesion. Consequently, wild type mice have a five-fold lower *H. pylori* colonization burden than *Muc1*$^{-/-}$ mice. Furthermore, human epidemiological studies have linked shorter Muc1 alleles to a higher probability of chronic gastritis progression, indicating that longer Muc1 alleles may confer a protective advantage to the host (565). Polymorphisms between human Muc1 alleles are largely restricted to the extracellular domain, which consists of a region of 30-90 tandem repeat units rich in serine and threonine. A study by Costa *et al.* (2002) demonstrated a significant positive association between the number of Muc1 tandem repeats and bacterial adherence for two strains of *H. pylori in vitro* (114). Longer Muc1 alleles probably evolved from shorter alleles via duplication events, and may have emerged to protect against pathogens such as *H. pylori* (568).

Complement cascade activation via the classical, lectin, and alternative pathways precedes the cleaveage of C3 convertase into C3a, an anaphylatoxin, and C3b, which binds to the surface of microbes (otherwise known as opsonization) to promote the eventual clearance of bacteria through phagocytosis. Additionally, C3 convertase may convert to the lytic C5 convertase through addition of a C3b molecule. Pathogens have evolved mechanisms to evade or block these processes (307). The *S. aureus* staphylococcal complement inhibitor (SCIN) protein stabilizes C3 convertase, preventing its cleavage into the active C3a and C3b fragments and attenuating anaphylatoxin activity

and bacterial opsonization (459). Like many of the previously described pathogenicity factors, the gene encoding SCIN (*scn*) is located on a PAI (460). Rather than preventing C3 cleavage, the *Neisseria meningitidis* serine protease NalP splits C3 at a unique site, generating shorter C3a-like and longer C3b-like fragments (126). The C3b-like fragments are capable of binding *N. meningitidis*, but are rapidly degraded by host complement factors H (fH) and I (fI). Although the activity of the C3a-like fragment has not been determined, this fragment lacks the conserved C-terminal arginine residue found in wild type C3a that is essential for activity, and therefore this truncated version is likely inactive.

A final example of an innate host selective pressure is the sequestration of host resources or nutrients away from colonizing bacteria. Iron, an essential nutrient, is in short supply within the host, either sequestered away in host cells or stored as a complex in hemoglobin, which is inaccessable to most microbes (502). Correspondingly, pathogens have been forced to develop numerous mechanisms to scavenge host iron. Predictably, these systems are often iron-regulated, and their genes expressed following bacterial exposure to the low-iron environment of the human host. Certain surface-bound receptors can recognize iron-bound complexes, such as heme, transferrin, or lactoferrin. Additionally, the secreted bacterial siderophores, aerobactin and enterobactin, steal iron away from host transferrin and lactoferrin. *E. coli* strains can encode for both of these systems (456). Another putative example of 'arms race' co-evolution is the mammalian neutrophil gelatinase-associated lipoprotein (NGAL). NGAL directly binds the catecholate-type ferric siderophore complexed to iron, preventing bacterial iron sequestration and eventually exerting a bacteriostatic effect upon microbial populations

(199). Some bacteria can even bypass this defense mechanism, however. Uropathogenic *E. coli* (UPEC) express the siderophore salmochelin, a glycosylated form of enterbactin resistant to the effects of NGAL (176).

If a pathogen manages to evade the innate immune system and can successfully compete with commensal microbes, it must then elude host adaptive immune responses, including B- and T-cell lymphocytes (252). One bacterial strategy employed inhibits lymphocyte proliferation. The VacA cytotoxin of *H. pylori* blocks the activity of host calcineurin, leading to downstream attenuation of interleukin-2 (IL-2) transcription, a key mediator of T cell proliferation (193). Alternatively, bacteria can avoid the adaptive immune response altogether by mediating lymphocyte cell death. For example, *Shigella* induces B-cell apoptosis through the actions of its type three secretion system (T3SS) (392).

Host selective pressures: Antibiotic resistance

The rise of adaptive antibiotic resistance in bacteria is perhaps one of the most intensely studied examples of pathogen evolution in response to a specific selective pressure(s) (59). Blair (2015) separates adaptive resistance mechanisms into three primary categories: reduced drug permeability through changes in the bacterial membrane or the development of efflux pumps, which quickly expel antimicrobials; mutations in antimicrobial targets to prevent binding; and the presence of enzymes that directly inactivate the antimicrobial agent itself (59). Well-characterized efflux pumps include the multi-drug exporters discovered in the common food-borne pathogens *E. coli* (ArcAB-TolC), *S. enterica* (EmrAB), and *S. aureus* (QacA/B, NorA) (24). Linezolid, an oxazolidinone class antibiotic, binds the 23S rRNA subunit and blocks tRNA interactions

with the A site to prevent peptide bond formation (585). Unsurprisingly, linezolid resistance in a number of bacterial species has been linked to a G2576T mutation in the 23S rRNA gene, precluding linezolid binding at this site and providing an example of the second category of adaptive drug resistance (169; 352). Finally, enzymes such as beta-lactamases, aminoglycoside acyltransferases, and monooxygenases are responsible for the inactivating hydrolysis, group transfer, or oxidation of their respective antibiotics (183; 589). The rapid spread of antimicrobial resistance, and the rise of multi-drug resistance, is often linked to the HGT dissemination of genes encoding these enzymes, as many PAIs and plasmids have been shown to carry one or more drug resistance genes (398). Resistance adaptations often come with a fitness cost, however, which has been demonstrated both *in vivo* and *in vitro* (25).

Microbial competition

Competition between microbes undoubtably plays a role in driving pathogen evolution, although this aspect of microbial evolution has not been widely studied and, except for a few examples, is still only very poorly understood. Bacteria can directly eliminate potential rivals through use of toxic peptides (bacteriocins) or through the utilization of type six secretion systems (T6SSs) (93; 435).

Bacteriocins, toxic peptides produced by bacteria, target and kill neighboring microbes. Colicins, the most well-known members of this category, are produced by strains of *E. coli*, although bacteriocins have been described in a wide variety of bacteria, including *S. aureus, Pseudomonas pyogenes, Yersinia pestis,* and *Serratia marcescens* (93; 188). In *E. coli*, colicins exhibit a number of different modes of action. Pore-forming colicins, such as colicin A, can insert into the inner membranes of susceptible bacteria to

create ion channels (137). Nuclease colicins, such as colicins E9 and E3, translocate across the outer and inner membranes of a susceptible bacterium to the cytoplasm, where they function as DNases (E9) or RNases (E3) (70; 561). Lastly, colicin M, a unique member of the colicin family, blocks peptidoglycan biosynthesis by degrading undecaprenyl phosphate-linked peptidoglycan precursors. These lipid-anchored intermediates are critical for the transport of peptidoglycan subunits across the cytoplasmic membrane (148; 228). To protect their own population against the harmful effects of these toxic peptides, the producers of colicins must concomitantly express immunity proteins. These immunity proteins block the action of their respective colicins. Pore-forming colicin immunity proteins sit in the inner membrane and block pore-forming colicins from inserting into the bacterial membrane. Nuclease colicin immunity proteins bind to DNase or RNase colicins to prevent their enzymatic activity, and the immunity protein Cmi binds colicin M to render it catalytically inactive (93; 402). Competing bacteria can acquire these immunity proteins via horizontal gene transfer (HGT), providing protection against *E. coli* colicin toxicity. For example, *Shigella*, which does not produce the pore-forming colicin V, nevertheless encodes an immunity protein on its SHI-2 PAI. This particular immunity protein, ShiD, protects against colicin V produced by strains of *E. coli* (195; 371).

The recently-discovered T6SSs of Gram-negative bacteria are responsible for the direct delivery of effector proteins into neighboring eukaryotic or bacterial cells, resulting in the death of host cells or the lysis of potential microbial competitors (465). VgrG1, an ADP-ribosyltransferase, is secreted from the *Aeromonas hydrophila* T6SS into host cells, where it disrupts the actin cytoskeleton and induces host cell apoptosis (518). Most of the

described T6SS effectors, however, have been shown to target other microbes. The T6S exported proteins 1 and 3 (Tse1 and Tse3) of *P. aeruginosa* exhibit amidase and muramidase activity, respectively, against bacterial peptidoglycan (463). *P. aeruginosa* also encodes type VI lipase effector (Tle) proteins, which degrade the bacterial phospholipid phosphatidylethanolamine (464). In *Dickeya dadantii*, the Rhs (rearrangement hotspots) proteins RhsA and RhsB are secreted through the T6SS and funtion as toxic endonucleases in susceptible bacteria. While *D. dadantii* is a plant pathogen, the human pathogen *S. marcescens* also expresses a T6SS-secreted Rhs-family protein, although its function is yet unknown (184; 299). Similar to the colicin proteins, pathogens that encode a T6SS must also express immunity proteins to prevent self-killing. *P. aeruginosa* encodes T6S immunity 1 and 3 (Tsi1 and Tsi3) proteins, which interact with and inactivate Tse1 and Tse3 through mechanisms that are not yet understood (463).

Intriguingly, T6SSs may also be effective tools for gene acquisition via HGT. In *V. cholerae*, the T6SS is co-regulated with competence genes by the regulator TfoX, and transformation events are dependent upon the presence of an active T6SS (71). Borgeaud *et al.* (2015) suggest that following activation of TfoX, both competence and T6SS systems are expressed and assembled. After T6SS-mediated lysis of neighboring cells, DNA is released to the extracellular space, where it can then transform the competent bacterium (71).

CONCLUDING REMARKS

Bacterial pathogens within the human host are exposed to a vast variety of different selective pressures that shape bacterial genomes and drive the evolution of novel

virulence factors. Concomitantly, human genomes also evolve as a result of these interactions, leading to a genetic ‘arms race’ between pathogens and their hosts. In bacteria, horizontal gene transfer (HGT) can enhance this process by allowing for the rapid dissemination of potentially beneficial alleles across strains or even species.

CHAPTER 3: *SHIGELLA*: VIRULENCE FACTORS AND PATHOGENICITY (61)

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K. A. Bliven and K. A. Lampel both contributed ideas for the general outline of the chapter. K. A. Bliven reviewed the literature and wrote the chapter, with the exception of the 'Adaptive immune system manipulation and evasion' and 'Drug resistance' sections, of which a rough draft was provided by K. A. Lampel. K. A. Bliven added to these sections and re-wrote portions to fit with the remainder of the chapter.

Note: The figure and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

Each year, *Shigella* species are responsible for 100 to 150 million infections worldwide, with nearly 500,000 of these infections occurring in the United States, where approximately 30% of cases can be attributed to the ingestion of contaminated foods. Clinical symptoms associated with shigellosis (otherwise known as bacillary dysentery) include diarrhea, sometimes bloody; fever; stomach cramps; and tenesmus. Possible sequellae of *Shigella* infection can include hemolytic uremic syndrome (HUS) or rheumatoid arthritis. This pathogen possesses a repertoire of virulence factors that modulate the host innate/adaptive immune systems and enable the bacteria to invade colonic epithelial cells, escape from phagocytic vesicles, and finally destroy mucosal cells lining the intestinal tract. One of the hallmarks of *Shigella* pathogenesis is utilization of the type three secretion system (T3SS) to insert bacterial effector proteins into host cells. Most of the genetic factors necessary for pathogenicity reside on a large virulence plasmid (pINV), while a handful of genes are found in chromosomal pathogenicity islands.

INTRODUCTION

Shigella is a Gram-negative, rod-shaped bacterium that causes shigellosis, a severe form of bacillary dysentery characterized by cramps, diarrhea (occasionally bloody), and fever. This pathogen is extremely effective at spreading via the fecal-oral route, with as few as 10 to 100 organisms required to cause disease (140). In the United States alone, the annual number of *Shigella* cases is roughly 500,000 (98); Scallan *et al.* suggest that around 30% of these are the result of contaminated food (98; 482). Globally, approximately 164.7 million diarrheal episodes are attributed to *Shigella* infection per

year (303). These infections result in 1.1 million fatalities, the majority of whom are children under the age of five. While outbreaks continue to occur in developed nations, *Shigella* puts a much higher burden on developing nations, where poor hygiene and water sanitation methods contribute to transmission. To compound this problem, persistent socioeconomic issues, such as childhood malnutrition, have been linked to an increased frequency and severity of diarrheal disease (215). Currently, there is no protective vaccine to prevent *Shigella* infection.

The *Shigella* genus is divided into four species or serogroups, each defined by a specific antigen type: *S. dysenteriae* (serogroup A), *S. flexneri* (serogroup B), *S. boydii* (serogroup C), and *S. sonnei* (serogroup D) (161). These organisms can be further differentiated into serotypes based on specific structural differences of the O-antigen lipopolysaccharide (21; 318). There are at least 43 distinct serotypes of *Shigella*, including 8 serotypes of *S. flexneri*, 19 of *S. boydii*, 15 of *S. dysenteriae*, and a single serotype of *S. sonnei*, which is considered to be clonal (98; 285).

Globally, *Shigella* species are unevenly distributed between the industrialized and developing world. In industrialized countries, *S. sonnei* contributes to 77% of all *Shigella* infections; in developing nations, *S. flexneri* is responsible for 60% of infections (303). Children in day care settings, or military/civilian travelers to endemic countries, are among the most at-risk populations for *Shigella* in industrialized countries (262; 303; 426). In contrast, displaced populations, driven by war, famine or persecution, are at highest risk for shigellosis in developing nations (303).

Shigella is distinguished from *Escherichia coli* only for medical and historical purposes; taxonomically, they are considered members of the same genus (437).

Pathogenic *Shigella* strains are postulated to have evolved directly from commensal *Escherichia coli* lineages between 35,000 and 270,000 years ago, and this evolutionary leap likely took place multiple times, each branch-off corresponding to the independent acquisition of a large 230 kB virulence plasmid (438; 599). Using comparative genomics, Lan *et al.* (2004) suggest that there are three main *Shigella* clusters, with clusters 1 and 2 containing strains that branched from *E. coli* 50,000 to 270,000 years ago, and cluster 3 emerging more recently, between 35,000 and 170,000 years ago (308). From this analysis, several outlier strains were also described, which possibly emerged independently of the three primary lineages. *S. flexneri* and *E. coli* K-12 genomes still share a common chromosomal backbone, which has undergone a number of large (>5 kb) rearrangements such as inversions and translocations (576). Because the majority of *Shigella* virulence determinants are plasmid-encoded, novel chromosomal ORFs (i.e., chromosomal genes unique to *Shigella* that are not present in *Escherichia coli*) are scarce. In *S. flexneri* 2a strain 2457T, only 175 ORFs are unique, while the remaining 3,030 ORFs are shared with *E. coli* K-12 strain MG1655 (576).

Two major events have driven the niche adaptation of *Shigella*: acquisition of the large virulence plasmid, encoding a type three secretion system (T3SS), and concomitant inactivation or loss of chromosomal factors. The addition of a functional T3SS drove these previously extracellular, commensal bacteria into the human colonic epithelial cell to exploit a novel intracellular niche (353; 477). In addition, several pathogenicity islands have been horizontally transferred to the chromosome, promoting virulence via genes that encode for aerobactin (iron) transport, O-antigen modification, and antibiotic resistance,

to name a few (259; 328; 485). These acquired virulence factors will be discussed in detail later in the chapter.

Conversely, genes no longer necessary for survival within the host cell were either lost or forced into a state of decay, resulting in a significantly smaller genome, and a remarkably high number of pseudogenes still remain in the *Shigella* spp. (170; 576). Feng *et al*. examined five *Shigella* genomes and discovered that 85 genes were commonly lost in all strains, while a staggering 1,456 genes were pseudogenized in at least one strain (170). Although a number of different mutational events are responsible for disrupting the *Shigella* genome, the majority of pseudogenes arose from either nonsense mutations or insertional inactivation of genes (317; 576; 603). Insertion sequence (IS) elements are abundant in the genomes of *Shigella* spp.: *S. flexneri* 2a alone has 314 IS elements, which is more than 7-fold that of related *E. coli* K-12 strains (273; 576; 603). Many of the inactivated or missing genes of *Shigella* spp. are from catabolic pathways (364). Most strains have lost the ability to grow on D-alantoin, D-malate, and xanthine as sole carbon sources, or inosine as a nitrogen source, even though the majority of *E. coli* strains readily utilize these nutrients. This inability suggests that the catabolic pathways to break down and utilize these substrates have been interrupted in *Shigella* through gene loss/inactivation. An example of a non-metabolic pathway in decay in the *Shigella* spp. is the flagellar system; as a result of deletions and/or inactivations of flagellar genes, *Shigella* strains are universally non-motile (170).

As *Shigella* evolved into a pathogen from *E. coli*, *Shigella* genes inhibitory to the new pathogenic lifestyle, known as antivirulence genes (AVGs), were also discarded (62; 342). The AVGs of *Shigella* include the genes that encode lysine decarboxylase (*cadA*);

quinolinate synthetase A/L-aspartate oxidase (*nadA*/*nadB*); spermidine acetyltransferase (*speG*); and an outer membrane protease (*ompT*) ((39; 171; 342; 346; 381; 434). Expression of these genes in a wild type *Shigella* strain attenuates different virulence phenotypes, such as cell-to-cell-spread (*ompT*) or macrophage survival (*speG*). Several of these genes encode for enzymes that catalyze an inhibitory end product. CadA, for example, catalyzes the conversion of lysine to the end product cadaverine, which, when added exogenously during *Shigella* infection, blocks PMN transepithelial migration, phagosome escape, and ShET enterotoxin activity (171; 342; 346). NadA/NadB, enzymes in the NAD biosynthetic pathway, produce quinolinic acid, which blocks invasion, cell-to-cell spread, and polymorphonuclear leukocyte (PMN) transepithelial migration (434). Alternatively, SpeG, a spermidine-acetyltransferase, depletes the bacterial cell of the beneficial substrate spermidine, which is critical for macrophage survival (39). In order to optimize pathogen fitness in the human host, the genes encoding these enzymes were inactivated or lost from all *Shigella* spp. (39; 62; 122; 342; 433).

Enteroinvasive *E. coli* (EIEC), which also harbors the large virulence plasmid encoding the T3SS, is often grouped with *Shigella* as a distinct pathovar within *E. coli* (420). Although EIEC strains are also invasive, they are considered less pathogenic, since they require a higher infectious dose, show a lower efficiency of cell-to-cell spread, and generate a milder host inflammatory response following infection (139; 368). EIEC are characteristically non-motile and have lost or inactivated many of the AVGs common to *Shigella*, including *cadA*, although some of the AVGs, such as *nadA/nadB* and *speG*, remain intact in at least a few EIEC strains (86; 92; 130). Moreover, EIEC share several biochemical properties with commensal *E. coli* that the *Shigella* spp. have lost, including

mucate and acetate production (498). These characteristics suggest that the EIEC pathotype represents an intermediate phenotype between commensal *E. coli* and pathogenic *Shigella* (308). However, EIEC strains are genetically less diverse than *Shigella*, and are thought to be more recent ancestors of commensal *E. coli*, suggesting that EIEC strains did not give rise to the *Shigella* spp. (308). Instead, shared niche adaptation is driving convergent evolution in the EIEC and *Shigella* lineages.

TYPE THREE SECRETION SYSTEM

Unique to Gram-negative bacteria, type three secretion systems (T3SSs) are specialized protein export systems utilized by bacteria to effectively exploit eukaryotic hosts (68; 107; 113). Bacterial adherence, invasion, and manipulation of the host's intracellular trafficking and immune systems are some of the diverse functions attributed to bacterial T3SSs. These processes are mediated by proteins known as effectors, which are translocated through the needle pore of the T3SS and deposited directly into the cytosol of the host cell, where they can interact with a target host protein(s). The targets of the T3SS proteins are incredibly diverse, and include host cell actin/tubulin, caspases, Rho GTPases, kinases, NF-kB, and cell cycle proteins, just to name a few (3). In *Shigella*, the T3SS facilitates bacterial invasion of the human colonic epithelium, enables subsequent cell-to-cell spread, and controls the host inflammatory response for the benefit of the bacterium (487) (Figure 1).

The T3SS needle apparatus is composed of 20-25 different proteins, which form three main components: a basal body with a multi-ring base to anchor the T3SS to the

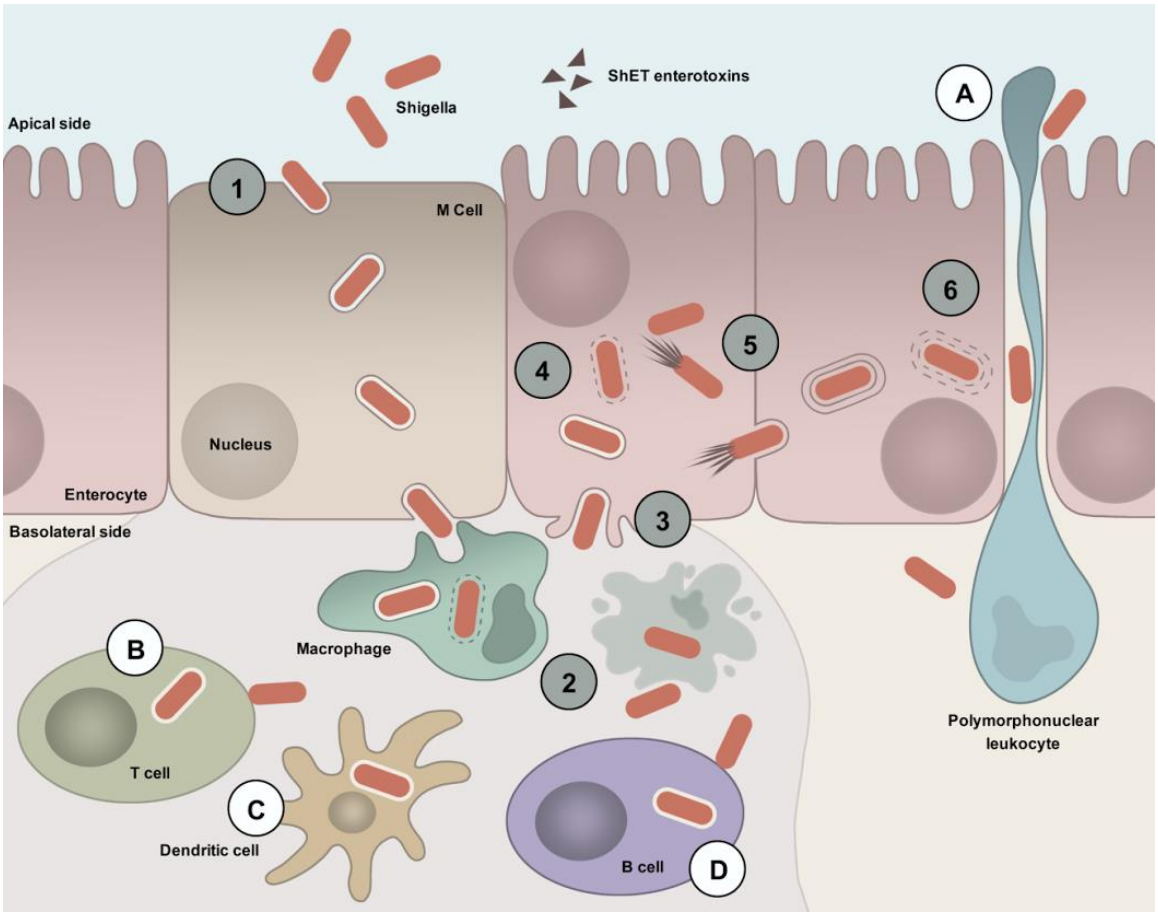

Figure 1. *Shigella* pathogenesis.
Following ingestion, *Shigella* travels through the gastrointestinal tract to the colon, where it invades the host epithelium. This multi-step process includes 1.) transcytosis through M cells; 2.) phagocytosis by macrophages, phagosome escape, and induction of pyroptosis; 3.) basolateral invasion of enterocytes; 4.) phagosome escape; 5.) actin polymerization and cell-to-cell spread; and 6.) lysis of the phagosome double membrane. Additionally, *Shigella* subverts the host immune system. A.) *Shigella* induces a massive polymorphonuclear leukocyte (PMN) influx, which is thought to disrupt tight junctions and provide bacteria an alternate route to access the basolateral membrane. B.-D.) *Shigella* also invades cells of the adaptive immune system, including T cells, dendritic cells, and B cells, to induce apoptosis. In T cells and B cells, T3SS needle contact with the host cell is sufficient for cell death. Details on these processes can be found in the text.

bacterial inner and outer membranes; a long needle-like portion that extends out from the bacteria and into the surrounding environment; and a translocon complex that creates pores in the eukaryotic cell membrane (153). Intriguingly, the T3SS structure bears a striking resemblance to the bacterial flagellum, and the core genes that form the T3SS needle share significant homology with core flagellar genes (1; 153; 204; 558). Both the T3SS and flagellum are complex structures that span the cytoplasmic and outer membrane of the bacterium, and both systems are driven by an F_0F_1-type ATPase (1; 412). Although it is widely accepted that these systems share an evolutionary heritage, debates continue over which system arose first, or whether they diverged from a common ancestor (1; 204). However, there are some obvious distinctions between the two systems. While the T3SS needle secretes bacterial proteins directly into eukaryotic host cells, the flagellar system functions as a key component of bacterial motility. Furthermore, T3SS genes are almost always clustered on single pathogenicity islands within the chromosome or on plasmids, which indicates a history of acquisition by lateral transfer (1). *Chlamydia* is a rare exception to this rule, as the *Chlamydia* T3SS genes appear to be distributed into at least four separate loci (1). Likewise, flagellar genes are always scattered in multiple operons throughout their respective bacterial genomes, making horizontal transfer of the system much less likely (323).

Genetics of the T3SS

The T3SS of *Shigella* is encoded on a large 230 kB virulence plasmid (pINV) present in all *Shigella* and Enteroinvasive *E. coli* (341; 477). Distinct variances of G/C content throughout the plasmid, coupled with the presence of three separate plasmid partitioning systems (two of which remain functional), suggest that the pINV is actually a

compilation of several ancestral plasmids (79). In addition to the T3SS structural, effector, and regulatory genes, this megaplasmid is also composed of over 90 insertion elements, more than 10 putative open reading frames (ORFs), and a host of plasmid maintenance, replication, segregation, and transfer genes (309). All virulent *Shigella* and EIEC strains carry one of two major variants of this plasmid: pINV A or pINV B (309). There are over 500 nucleotide polymorphisms between the two variants, with over 300 of these changes resulting in an amino acid difference. It is not yet known if these variants are responsible for differences in pathogenesis between strains.

The genes that encode the T3SS structural needle are grouped together on pINV in a 32 kB cluster known as the *Shigella* Entry Region (SER) (340; 480). This cluster contains the *ipa*, *mxi*, and *spa* operons necessary and sufficient for construction of the T3SS needle and the initial invasion process. Outside of the SER, there are over 20 genes that encode effectors known or suspected to be secreted through the T3SS.

Structure of the T3SS and recognition of secreted effectors

As mentioned above, the *Shigella* T3SS consists of three components: the basal body, the needle, and the translocon tip complex. The portion of the basal body embedded in the outer membrane of the bacteria consists of the MxiD ring and the stabilization protein MxiM; MxiJ and MxiG form a ring through the inner membrane (489). The cytoplasmic portion of the basal body includes a C-ring platform (Spa33, Spa47, and MxiN) essential for the recognition, sorting, and secretion of effectors through the type three needle (257; 311). One of these C-ring proteins, Spa47, functions as the T3SS ATPase, and provides the energy necessary for effector transport across the bacterial membrane (147). A nonameric ring MxiA sits between the T3SS needle pore

and the ATPase, and facilitates the secretion of T3SS effectors, potentially with the assistance of Spa13 and MxiC (5; 26; 104; 337). Spa32, which controls the length of the T3SS needle and interacts with the assembly protein Spa40, is likely located near the base of the needle complex (72; 532). Other T3SS proteins, including Spa9, Spa24, and Spa29 are also proposed components of the basal body, although their structural locations and functions have not yet been determined (67).

The T3SS needle is composed of the major MxiH subunits, which assemble in a helical fashion to form the cylindrical needle, and the minor subunit MxiI, which is hypothesized to form a T3SS needle cap comparable to the flagellar hook cap FlgD (66; 112). The precise transport and assembly of MxiH and MxiI appear to be dependent on the basal body proteins MxiK, MxiN, and Spa47, as mutants defective in any of these three genes produce non-functional T3SSs lacking the needle component (279).

At the needle tip, the translocon complex (IpaD, IpaB, and IpaC) mediates the recognition of environmental sensors required for activation of the T3SS, and participates in the insertion of IpaB and IpaC into host membranes (563). IpaD is a hydrophilic protein bound via its C-terminal end to the tips of T3SS needles (156). Four IpaD units are thought to bind to each tip; in the inactive state, these proteins block secretion through the needle pore (132; 563). IpaB and IpaC are only recruited to the tip complex following the interactions of IpaD with a secretion trigger, such as exposure to bile salts (132).

An N-terminal signal (~20 residues of the protein sequence) is utilized to direct T3SS effectors to the base of the needle for subsequent secretion, although a strong consensus sequence or structure has not yet been identified and these signals may be specific to certain T3SSs (229; 470). Because of the narrow confines of the needle pore

(~2-3 nm diameter), T3SS effectors are shuttled through in a partially unfolded state, and must be re-folded when they reach the host cell cytoplasm (14; 65; 66). Certain chaperone proteins help preserve the unfolded state of these proteins as they pass through the T3SS; however, not all bacterial effectors have chaperones (511). The *Pseudomonas* effector AvrPto, for example, utilizes a pH-sensitive mechanism for proper unfolding and re-folding (121).

In *Shigella,* IpgA, IpgC, IpgE, and Spa15 function as the T3SS chaperone proteins. IpgA chaperones IcsB; IpgC chaperones IpaB and IpaC; IpgE chaperones IpgD; and Spa15 chaperones at least IpgB1, IpgB2, IpaA, OspC3, and OspD1, and possibly OspB, OspC2, and MxiE as well (219; 390; 400; 408; 410; 415).

Regulation of the T3SS

Optimal expression of the *Shigella* T3SS is dependent on several factors that prevent expression of this system outside the host to ultimately conserve energy. One of these components of regulation is temperature (343). H-NS, a global regulator in *Escherchia* and one of the main components of bacterial chromatin, is a critical repressor of many of the T3SS genes in *Shigella*, including the two transcriptional activators, *virF* and *virB* (136; 540). In conditions designed to mimic temperature outside the host (30° C), H-NS binds weakly to curved sections of DNA in the promoter regions of numerous T3SS operons, including *virF*, *virB*, *ipgD*, *spa*, *icsP*, *icsA*, and *virA*, repressing transcription (47; 97; 109; 432). Upon exposure to host temperature (37° C), H-NS binding is postulated to weaken due to thermally-induced changes in the DNA supercoiling of these promoter regions, and the repressor can be displaced by other transcriptional activators, such as the factor for inversion stimulation (FIS) and the

integration host factor (IHF), which bind to and activate the *virF* promoter (162; 428; 541).

Once activated, expression of the T3SS is dependent upon a cascade of events (136). VirF, the primary transcriptional activator of the system, is a member of the AraC family of transcriptional regulators (190). Once expressed, VirF binds to and activates the promoters of *virB* and *icsA* to initiate this regulatory cascade (540). VirB, a secondary regulator of the *Shigella* T3SS, is then available to activate the *ipa*/*mxi*/*spa* promoters (572). The *ipa* (invasion plasmid antigens) operon consists of *ipaBCDA*, several *ipg* genes (*ipgCBA*), *icsB*, and a putative acyl carrier-encoding gene, *acp*; the *mxi* (membrane expression of ipa) operon contains the remaining *ipg* (*ipgDEF*) and *mxi* genes (*mxiGHIJKLMEDCA*); and the *spa* (surface presentation of ipa) operon carries the nine *spa* genes (*spa15*, *spa47*, *spa13*, *spa32*, *spa33*, *spa24*, *spa9*, *spa29*, and *spa40*). As mentioned previously, expression of these three operons is both necessary and sufficient for the assembly of the *Shigella* T3SS and initial entry into the host cell. VirB also transcriptionally activates the first set of T3SS effector genes, including *ospC2*, *ospC3*, *ospC4*, *ospD1*, *ospD2*, *ospZ*, and *icsP* (42; 97; 313). Presumably, these effectors must be stored prior to secretion so that the bacteria are primed for the early stages of host cell survival and immune system modulation. MxiE activation, which drives the expression of a second set of T3SS effector genes, is blocked by the antiactivator complex OspD1 and Spa15 until secretion is induced (415). This second set of genes includes *ospD3*, *ospE1*, *ospE2*, *ospG*, *ipaH1.4*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8* (313). Finally, in the absence of secretion, *ospF*, *ospC1*, *ospB*, *phoN2*, and *virA* are driven by VirB activation; in contrast, during secretion, these genes are activated in a MxiE-dependent fashion.

Osmotic stress, pH, and the availability of oxygen also affect T3SS expression (335; 361; 382). In conditions of low osmolarity, the RNA chaperone Hfq binds to and decreases the stability of *virB* mRNA, and corresponding T3SS expression is decreased (361). In response to environmental pH, expression of *virF* is attenuated at pH 6.0 and activated at pH 7.4 (382). The role of a two-component regulatory sensor protein (CpxR) in this regulation is recognized but not well understood. Lastly, oxygen also modulates regulation; in the presence of oxygen, FNR, a regulator of anaerobic metabolism, dissociates from the promoters of the T3SS genes *spa32* and *spa33*, allowing transcription to proceed (335). Spa32, a component of the T3SS critical for controlling needle length, and Spa33, a regulator of Ipa translocation, are both critical for effector secretion (490; 532). Levels of oxygen in the intestinal tract vary depending upon site, and it is likely that sufficient oxygen required for FNR disassociation would only be available close to the mucosal surface, promoting T3SS secretion and priming the bacterium for impending invasion (335; 547).

Adherence and invasion

The human colonic epithelium presents a significant challenge to adhesive and invasive pathogens. This barrier consists of a thick layer of gastric mucosa, which covers a single layer of epithelial cells knit closely together by tight junctions, with a limited repertoire of ligands present on the apical surface for bacteria to bind. To circumvent this problem, *Shigella* preferentially invades host epithelial cells through the basolateral surface, and employs at least two methods to gain access to this membrane (373; 422; 612). First, *Shigella* transcytose through specialized microfold (M) cells, which overlay organized colonic lymphoid follicles harboring B lymphocytes, macrophages, and

follicular dendritic cells. In the normal course of sampling the intestinal microbiota, M cells endocytose or phagocytose viruses, macromolecules or even entire microbes from the intestinal lumen and trancytose them to the lymphoid tissue underneath. There, the antigens are engulfed by resident macrophages or dendritic cells and then presented to T cells to stimulate either tolerance or immunity (280). Because of their vital immunological role in routine antigen-sampling, M cells express a variety of unique ligands that are absent on the apical surface of other intestinal epithelial cells, including glycoprotein 2, uromodulin, and ANXA5 (330). Several of these receptors directly bind bacterial adhesins; glycoprotein 2, for example, associates with FimH of type I pili and is essential for M cell transcytosis in certain Gram-negative bacteria (232). Consequently, while M cells are clearly necessary for passive immunological surveillance of the intestine, they actually represent a weak point in the host defense that can be exploited by any bacterial pathogen able to avoid or overcome the host immune responses of the underlying lymphoid tissue (275).

During infection with *Shigella*, or other pathogens such as *Salmonella* and *Yersinia*, M cell transcytosis is actively initiated by the bacterium, as mutants defective in adhesion or invasion are attenuated in M cell transport (276; 333; 476). *Shigella* ligands are hypothesized to bind specific M cell glycoprotein receptors in order to facilitate uptake, although specific receptors have not yet been identified (478). Following M cell entry, *Shigella* does not lyse the endocytic vacuole nor induce immediate cytotoxicity of the cell; instead, the bacterium is released directly into the intercellular space of the intraepithelial pocket (476).

Shigella are then engulfed by resident macrophages and dendritic cells of the intestinal lymphoid tissue, and quickly escape into the host cytoplasm (142; 613). In macrophages, the *Shigella* T3SS effector IpaB binds to caspase-1 (previously known as interleukin-1 beta converting enzyme), which is responsible for processing the pro-inflammatory cytokine interleukin-1 beta (IL-1β) to its biologically active form, rapidly inducing cell pyroptosis (103; 538; 611). In dendritic cells, *Shigella* also induce cell death, but the mechanisms involved are less clear, partially involving caspase-1 and as-yet-unidentified cysteine protease(s) (142). Additionally, *Shigella* may invade B lymphocytes and induce cell death, while extracellular *Shigella* kill B lymphocytes shortly after interaction of the needle tip complex protein IpaD with Toll-like Receptor 2 (TLR2) (392). Following pyroptosis, the bacteria are free to invade the basolateral membrane of the colonic epithelium. Once inside, the bacteria hijack host cell pathways to amplify PMN influx across the intestinal barrier, further destabilizing the epithelium and promoting bacterial translocation (425).

The *Shigella* invasive process is T3SS-dependent. First, the needle contacts host cell lipid rafts, cholesterol/sphingolipid-rich regions that cluster closely together in the eukaryotic membrane (306; 557). The formation of the needle tip complex and membrane insertion of the *Shigella* effectors required for invasion occur in a stepwise manner. IpaD, at the T3SS needle tip, is thought to act as the environmental sensor for invasion. Following stimulation with deoxycholate or other bile salts during bacterial passage through the small intestine, IpaD recruits IpaB to the T3SS needle complex (131; 132). During initial contact with the host cell, IpaB is inserted into the phospholipid membrane where it binds directly to the transmembrane hyaluronan receptor, CD44. On the

cytoplasmic side, CD44 is associated with the actin cytoskeleton, and therefore may be critical for bacterial hijacking of host cell signaling cascades (7; 245; 306). *Shigella* T3SS adherence has also been shown to initiate further accumulation of cholesterol and raft-associated proteins at the site of entry, likely drawing in the necessary components and cytoskeletal elements required for invasion, such as the sorting protein p4.1 (306; 461). Finally, IpaC is recruited to the needle tip following IpaB interaction with host lipid rafts, and then inserted into the host membrane (152). An actual trigger mechanism for effector translocation remains poorly understood, however. Congo Red, a synthetic sulfonated azo dye, induces type three secretion *in vitro*, and it is hypothesized that interactions between the needle and either Congo Red or sphingolipid rafts will destabilize the needle tip complex to open the pore for secretion (36).

Reorganization of the host cytoskeleton induces the cell to engulf the bacterium, a process known as membrane ruffling (387). Following secretion, the released IpaB, IpaC, and IpaD effectors bind $\alpha_5\beta_1$ integrin, which prompts additional actin cytoskeleton rearrangement (573). The *Shigella* effector IpaC triggers actin polymerization and formation of filopodial and lamellipodial extensions by activating the small GTPases Cdc42 and Rac1, respectively. This process is likely initiated through induction of the Abl/Arg tyrosine kinase signaling pathway (226; 387; 549). Other T3SS effectors also play a role in the activation of Cdc42 and Rac1. IpgB1 and IpgB2 directly drive the activation of both small GTPases by stimulating the dissociation of guanosine disphosphate (GDP) to allow for guanosine triphosphate (GTP) binding (146; 295; 401). Activated Cdc42 and Rac1 induce members of the Wiskott-Aldrich syndrome protein (WASP) verpolin-homologous family (known as WAVE proteins) to recruit the actin-

nucleating complex Arp2/Arp3 for cytoskeletal remodeling (146). To uncouple the eukaryotic plasma membrane from the actin cytoskeleton and facilitate the underlying rearrangements, the *Shigella* inositol 4-phosphatase IpgD dephosphorylates phosphatidylinositol 4,5-bisphosphate into the lipid phosphatidylinositol 5-monophosphate, resulting in destabilization of the plasma membrane composition (389). Finally, IpaA directly binds to the N-terminal residues of a focal adhesion protein, vinculin, and stimulates an association between vinculin and F-actin (413; 548). The interactions between vinculin, IpaA, and F-actin promote actin depolymerization, further organizing the cell surface for efficient invasion (74). Unlike IpaB/C/D, however, IpaA is not essential for cell entry; a mutant deficient in IpaA is still invasive, although its invasion efficiency is slightly attenuated compared to the wild type parent (548).

Once inside the epithelial cell, the bacterium must lyse the endocytic vacuole to escape into the host cytoplasm for subsequent bacterial replication and dissemination, and to avoid death following lysosome fusion with the endocytic vacuole. Thus far, studies on *Shigella* endocytic/phagocytic vacuole escape have focused on the phenotype in macrophages rather than epithelial cells, although the mechanisms are likely similar. Within 30 minutes following infection, almost half of *Shigella*-infected macrophages have undergone endocytic vacuole rupture (494). Using a tightly-regulated expression system, Page *et al.* were able to uncouple *Shigella* invasion from post-invasion processes to demonstrate that the T3SS effectors IpaB and IpaC, in addition to the cytoplasmic chaperone IpgC, are essential during bacterial escape from the phagosome in macrophages (409). IpaC binds to and destabilizes phospholipid membranes, while IpaB forms cation pores that promote potassium flux into endosomes; the resulting disruption

is likely the primary mechanism for endosomal/phagosomal lysis (123; 494). Intriguingly, IpaC may have two separate mechanisms during invasion and phagosomal escape (407). In *Salmonella*, the IpaC homolog, SipC, is essential for invasion; however, in contrast to IpaC, SipC possesses no endocytic lysis activity (405). When *ipaC* and *sipC* are swapped in *Shigella* and *Salmonella* strains, *Shigella* expressing *sipC* lose the ability to escape the phagocytic vacuole, which the *ipaC*-expressing *Salmonella* lyse. Furthermore, insertional mutagenesis studies of IpaC by Bârzu *et al.* suggest that different domains of IpaC may be responsible for the distinct phenotypes of invasion and vacuole escape (41).

Intracellular motility and intercellular spread

Motility of *Shigella* is mediated by IcsA, an outer membrane protein exported across the cytoplasmic membrane via the Sec secretion system, and autotransported across the outer membrane (53; 76; 201; 206). Following secretion, IcsA localizes to a single pole on the bacterium, a process which is aided by the protease IcsP (formerly known as SopA), which cleaves IcsA from the entire bacterial surface (144; 512). Steinhauer *et al.* propose that IcsA is targeted to the pole for insertion, but diffuses towards the septum as it accumulates (512). IcsP, meanwhile, slowly cleaves IcsA equally across the bacterial surface. The disparity in IcsA concentration between the pole and the remainder of the bacterium, coupled with the slow catalytic activity of IcsP, ensures that IcsA is prevented from accumulating at non-pole regions. The chaperones DegP, Skp, and SurA, as well as the periplasmic apyrase PhoN2, are also important for proper IcsA localization, although the mechanisms involved are not yet clear (439; 493).

Once at the pole, IcsA recruits and activates the neuronal Wiskott-Aldrich syndrome protein (N-WASP), enhancing N-WASP affinity for the Arp2/Arp3 complex, which then switches to its active form (145; 200; 525). The activated Arp2/3 complex binds G-actin, and an actin tail is polymerized at the bacterial pole to allow for motility. Similar to *Listeria monocytogenes*, *Shigella* bacteria can move through the host cytoplasm at rates of approximately 26 μm per minute (205). Additionally, host protein kinase C (PKC) activation is essential for the organization of actin stress fibers to promote cell-to-cell spread during *Shigella* infection; the *Shigella* T3SS effectors OspE1 and OspE2 interact with host PDZ/LIM domain protein 7 (PDLIM7) to activate PKC (125; 600).

Shigella specifically targets tight junctions between host cells for spread (187). Host cell pseudopodia containing *Shigella* are engulfed by neighboring cells through a clathrin-mediated endocytic pathway dependent upon host clathrin, Epsin-1, and Dynamin-2. Tricellulin, a tight junction integrity protein that localizes primarily to tricellular tight junctions, is an essential component of this process. Finally, the Vps/VacJ ABC transporter system encoded on the bacterial chromosome also appears to play a role in vacuole lysis and/or cell-to-cell spread, possibly through maintaining lipid asymmetry in the outer membrane or by helping to target the tricellulin-containing epithelial cell junctions, although the exact mechanism has not yet been elucidated (91). Once inside the new host cell, the bacterium must lyse the double host membrane, a process dependent upon the T3SS effector proteins IpaB, IpaC, and IpaD (491).

HOST IMMUNE SYSTEM MANIPULATION AND EVASION

Humans are the only known host for *Shigella* species, although there have been reports of higher primates infected with the pathogen after close contact with zookeepers (38). Patients with shigellosis typically recover within several days, but if the infection goes untreated, severe cases can progress, and patients may present with bloody, mucoid stools. Patients infected by Shiga toxin-producing strains may also be forced to endure additional complications such as hemolytic uremic syndrome, hemorrhagic colitis, or rheumatoid arthritis (50). Sometimes, the infection is fatal.

Following ingestion, *Shigella* must cope with a number of innate host defenses, such as the acidic environment of the stomach, the competing commensal microbiota of the gastrointestinal tract, and the nearly impenetrable barrier established by colonic epithelial cells. Moreover, subsequent bacterial invasion of the host epithelium provokes a number of host innate and adaptive immune responses. A classic and impressive example of a *Shigella*-induced host defense is the substantial polymorphonuclear leukocyte (PMN) influx to the site of infection, which eventually leads to tissue destruction and concomitant shedding of the pathogen to clear the infection (338; 422). Host adaptive immune responses also assist in recognizing and destroying the pathogen (446). In an attempt to circumvent these outcomes, *Shigella* has developed mechanisms to manipulate or evade the host immune system.

Innate immune system manipulation and evasion

Successful manipulation of the innate immune system is critical to the survival of *Shigella* within the host. As previously mentioned, *Shigella* directly induces the PMN influx, a massive inflammatory response that is one of the hallmarks of *Shigella* infection in the gut (425). This influx is likely a critical factor to destabilize the integrity of the

colonic epithelium, and eventually provide bacteria with a route to the basolateral membrane, where they can preferentially invade (422). However, PMNs are also detrimental to bacterial infection, as they can devour and kill extracellular *Shigella* (326; 579). Invading bacteria must therefore carefully manage the host inflammatory response: too little inflammation, and translocation of *Shigella* across the intestinal epithelium might be attenuated; too much inflammation, and *Shigella* might be overwhelmed and killed by the immune system. An optimal level of inflammation, therefore, is critical for successful colonization and dissemination. A number of early and late *Shigella* T3SS effectors are secreted into the host cell with the sole purpose of supporting this complex interplay.

The rapid induction of inflammation is due to several key *Shigella* proteins, including the T3SS effectors OspB and IpaH7.8, and SepA, a serine protease (23; 48; 523). In *Shigella*-infected macrophages, IpaH7.8 targets the host protein glomulin for degradation, activating the inflammasome and inducing pyroptosis within 2-3 hours post-infection (523). The bacterial-induced macrophage death releases the cell's stores of mature IL-1β, which instigates a massive influx of PMNs to the tissue underlying the intestinal epithelium, although hepoxilin A_3 is required for the final induction of PMNs across the epithelium itself (375; 479). OspB activates host ERK1/2 and p38 mitogen-activated protein kinases (MAPKs), which in turn phosphorylate and activate cytosolic phospholipase A2 (cPLA2), an enzyme that releases arachidonic acid from phospholipid membranes (23; 375; 377). Arachidonic acid is converted to hepoxilin A_3, the chemoattractant required during *Shigella* infection to draw PMNs through the colonic

epithelium (375). The autotransporter SepA functions as a cysteine protease to induce tissue destruction and inflammation in a rabbit ileal loop model of *Shigella* infection (48).

Meanwhile, other T3SS effectors, including members of the Osp and IpaH families, play critical roles in dampening or tempering the host inflammatory response (294). The IpaH proteins are a class of E3 ubiquitin ligases, and this family includes five genes on the virulence plasmid (*ipaH1.4*, *ipaH2.5*, *ipaH4.5*, *ipaH7.8*, and *ipaH9.8*), and seven additional *ipaH* family genes (*ipaH0722*, *ipaH0887*, *ipaH1383*, *ipaH1880*, *ipaH2022*, *ipaH2202*, and *ipaH2610*) on the bacterial chromosome (31; 458; 501). Not all *Shigella* strains carry a complete set of functional *ipaH* genes, however; for example, in *S. flexneri* YSH6000, *ipaH1.4* and *ipaH2.5* are pseudogenes (31). Furthermore, of the chromosomal *ipaH* genes, only four are presumed intact in *S. flexneri* strain 2457T, as the remaining three are disrupted by insertion elements or frameshift mutations (576). Each of these genes shares a highly conserved C-terminal region required for E3 ligase activity, as well as a more variable N-terminal region containing a common leucine-rich repeat motif essential for substrate recognition (501; 608). Therefore, although all IpaH proteins presumably function as ubiqutin ligases to target certain host cell proteins for degradation, each has a specific target; IpaH7.8 targets glomulin; IpaH9.8 targets NEMO; IpaH4.5 targets the p65 subunit of NF-κB; and IpaH0722 targets TRAF2 (29; 30; 523). IpaH9.8, IpaH4.5, and IpaH0722, which tag proteins of the NF-κB signaling cascade for degradation, contribute to the attenuation of NF-κB activation and suppress the host inflammatory response. In addition to the IpaH proteins, members of the Osp (outer *Shigella* protein) family also contribute to the attenuation of the NF-κB signaling pathway. OspF is a phosphothreonine lyase that irreversibly dephosphorylates host

ERK1/2, p38, and JNK MAPKs; the activity of this T3SS effector directly opposes that of OspB (319; 452). Intriguingly, although OspF and OspB appear to follow the same initial time course of expression and secretion, expression of *ospB* decreases 1 hour post-infection, with *ospF* expression remaining more stable, indicating that perhaps OspB plays a predominantly early role with OspF tempering the inflammatory response following initial infection (23). OspI binds host Ubc13, an E2 enzyme, and deamidates Gln100 to a glutamic acid residue (186; 471). The altered Ubc13 is unable to polyubiquitinate TRAF6, and downstream NF-κB activation is attenuated. OspG functions as a serine-threonine kinase that binds to E2-ubiquitin complexes such as Ub-UbcH5b, which is an important factor for the ubiquitination and degradation of phospho-IκBα (293). Although the exact mechanism has not been elucidated, OspG binding to Ub-UbcH5b prevents phospho-IκBα degradation and corresponding NF-κB activation. Finally, OspZ, like its homologs NleE and NleB in enteropathogenic *E. coli* (EPEC), also attenuates IκB degradation (386).

Inflammation is not the only host process influenced by *Shigella* proteins. The OspC family of proteins (OspC1, OspC2, OspC3, and OspC4) are thought to play roles in the inhibition of different caspases within the host cell, likely delaying epithelial cell death and prolonging time for bacterial replication and spread (297). OspC3, the only one of these effectors to be characterized thus far, binds directly to the p19 subunit of caspase-4, inhibiting the interaction of the p19 and p10 subunits required for heterodimerization and activation of caspase-4. Another T3SS effector, IcsB, binds cholesterol to protect the actin-polymerization protein IcsA from stimulating host autophagy (289).

Adaptive immune system manipulation and evasion

In addition to subverting the innate immune system, *Shigella* also molds adaptive immune responses to promote pathogenesis. Both T cells and B cells are manipulated to help *Shigella* evade clearance by the host immune system.

Salgado-Pabón *et al.* recently reviewed the literature on the role of T lymphocytes during *Shigella* infection (469). T cells locally present at the site of infection may be hijacked in one of two ways: *Shigella* can either invade T lymphocytes or utilize the T3SS to inject effector proteins directly into these cells in the absence of bacterial invasion (298). As part of *Shigella*'s defense mechanism, both *in vitro* and *in vivo* experiments indicate that the pathogen restricts the migration of activated $CD4^+$ T cells. The PAI-encoded ShiA protein plays a role in attenuating this process although the mechanism is not yet known (266). The T3SS effector, IpgD, has also been identified as a critical component of this inhibition (298). IpgD mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) to phosphatidylinositol 5-monophosphate (PI5P) at the plasma membrane, decreasing the concentration of phosphorylated ERMs (ezrin, radixin, and meosin proteins). In the absence of activated (phosphorylated) ERMs, T cells are unable to migrate in response to chemoattractants, such as the pro-inflammatory cytokine surge resulting from *Shigella* invasion. In addition, the ability of $CD8^+$ T cells to stimulate the adaptive immune system is muted during invasion by *Shigella* species through an unknown mechanism (271). Lastly, dendritic cells, which act as antigen-presenting cells during infection, have a reduced level of recruitment upon *Shigella* invasion (509).

As mentioned previously, Nothelfer et al. observed B lymphocyte death, both *in vitro* and *in vivo*, that were dependent upon the presence of IpaD (392). Toll like

receptors (TLR), specifically TLR2, can interact with IpaD and as yet unidentified bacterial co-signals to induce B cell apoptosis.

SHIGA TOXIN

Shiga toxin (Stx) was named after Dr. Kiyoshi Shiga, who identified *Shigella* as the causative agent of bacillary dysentery in 1898 (550). Shortly after this discovery, Conradi and Shiga/Neisser independently described the deleterious effects of Stx in a rabbit model (178). Another century would pass before Stx, and the related Stx-like enterotoxins of enterohemorrhagic *E. coli* (EHEC), would be epidemiologically linked to and considered necessary for the development of haemorrhagic colitis and haemolytic uremic syndrome (HUS) in humans (180; 284; 286; 292; 394; 445).

For decades, Stx was thought to be confined to strains of *S. dysenteriae* type 1, only rarely appearing in isolates of *S. dysenteriae* type 4 or *S. sonnei* (54; 216; 516). Between 2001-2003, however, 26 *S. flexneri* isolates harboring Stx were cultured from patients around the globe, many with a history of recent travel to Haiti or the Dominican Republic (208). The emergence of Stx-producing non-*S. dysenteriae* 1 *Shigella* strains has become a serious health concern, particularly because the bacteriophage carrying the Stx genes in *S. flexneri*, ΦPOC-J13, is novel among Stx-carrying bacteriophages. In *S. dysenteriae* 1, the functional Stx genes are nestled within a defective lamboid prophage that was likely inactivated through numerous IS element insertions and rearrangements (347). In the Shiga toxin-producing *E. coli* (STEC) strains, the Stx-like toxins Stx1 and Stx2 are carried on a diverse number of lambdoid phages (378). One of these phages, 933W, appears to be closely related to the 7888 phage carrying Stx in *S. sonnei* (427; 516). The emergence of bacterial strains carrying novel toxin-encoding phages suggest

that there may be an environmental reservoir for such phages, and integration events into new *Shigella*/*E. coli* strains may continue to occur.

The two structural genes encoding Stx subunits, *stxA* and *stxB*, are expressed as a single transcript, although there is an additional promoter directly upstream of *stxB*, which suggests this gene may also be independently transcribed (218; 395). Although toxin regulation in STEC has been well studied, considerably less attention has been given to Stx regulation in *S. dysenteriae* 1 (124; 376; 406). The promoter of the *stxA*/*stxB* operon is negatively regulated by the ferric uptake regulatory protein (Fur); in the presence of iron, the Fur-iron complex binds upstream of *stxA*/*stxB* to repress expression, which suggests that this operon is likely expressed in the host tissues, where free iron availability is limited during infection (90; 527; 578).

Shiga toxin is an A_1B_5 holotoxin; the enzymatically active 32 kDa A-subunits are C-terminally anchored to a pentameric ring of 7.7 kDa B-subunits, each of which have three binding sites for the host receptor, which is predominantly the glycosphingolipid receptor Gb3 (51; 267). Stx toxin export or release from the bacteria is not yet well understood, although outer membrane vesicle formation may be important for this process (141).

Gb3 is highly expressed in vascular endothelial cells and the kidney, two prominent sites of Shiga toxin damage. Several renal cell types, including proximal/distal renal tubule cells, mesangial cells, and glomerular epithelial cells, carry the receptor (355). Once internalized into the host cell, the enzymatically active A-subunit is cleaved by host furin protease under low pH conditions into A1 and A2 fragments, which remain attached by a single disulfide bond (191). Following cleavage, the fragments dissociate

in the endoplasmic reticulum and the A1 subunit is transported to the cytosol, where it removes a single adenine base from the host 28S rRNA within the 60S ribosomal subunit (151). Disruption of host ribosomal activity renders protein synthesis impossible, and cell death shortly follows.

SHIGELLA PATHOGENICITY ISLANDS

The acquisition by horizontal gene transfer of pathogenicity islands (PAIs) is a significant factor driving microbial evolution, producing pathogens from commensals and pushing pathogens into novel niches or helping them overcome host defenses or antibiotic treatment (484). These genetic islands are typically characterized by the presence of one or more associated virulence genes, G+C content atypical of the chromosome, and a close proximity to mobile genetic elements, such as integrases or insertion sequence (IS) elements, which suggest horizontal transfer of the DNA from an outside source (220; 484).

Although *Shigella* virulence is largely attributable to the T3SS of the large virulence plasmid, there are several *Shigella* PAIs that have been identified on the chromosome: SHI-1, SHI-2, SHI-3, SHI-O, and SRL (487). It is important to note that these PAIs are not universally present in *Shigella*. The SHI-1 locus, for example, is entirely absent from *S. dystenteriae* strain Sd197 and *S. flexneri* strain Sf8401, and other *S. boydii* and *S. sonnei* strains are missing the *pic*/*set1A*/*set1B* genes of the PAI (388; 597).

SHI-1 locus

SHI-1, previously known as the *she* locus, is flanked by a complete *pheV* tRNA gene directly upstream and a 22 bp repeat of *pheV* downstream (16). This is perhaps

unsurprising, as the *pheV* gene is a common site of insertion for PAIs and transposons in both *E. coli* and *Salmonella*, and tRNA genes have been shown to be effective insertion points (221; 529). Four characterized genes (*set1A*, *set1B*, *sigA*, and *pic)* and 28 putative open reading frames (ORFs) form this locus. All four characterized genes encode virulence factors that are optimally active at 37°C, mimicking *Shigella* T3SS temperature-dependent expression (16; 45; 239).

The *set1A/set1B* genes encode an A_1B_5 enterotoxin, ShET1, which is highly homologous (99%) to the virulence plasmid-encoded ShET2 and also contributes to the onset of watery diarrhea during shigellosis (165; 166). The other two characterized genes of the SHI-1 locus, *sigA* and *pic*, encode autotransporters. SigA, an immunoglobulin A-like cytopathic protease, is cytotoxic to HEp-2 cells and can degrade casein *in vitro*, although its target substrate in the host is not yet known (16; 443). A Δ*sigA S. flexneri* 2a isolate is significantly impaired in its ability to induce fluid accumulation in the rabbit ileal loop model, indicating that SigA likely plays a role during the watery phase of diarrhea (16). Finally, *pic* encodes a serine protease that binds host mucin to catalyze its degradation (217; 239). This breakdown process aids in destroying the thick mucus layer that lines the intestine, likely promoting *Shigella* colonization and invasion.

Of the remaining 28 putative open reading frames (ORFs) in SHI-1, most share sequence homology with hypothetical bacteriophage-associated genes, and their functions have yet to be elucidated. However, one of the ORFs, termed *sap*, bears high sequence homology to the previously characterized Ag43, an autotransporter surface protein responsible for autoaggregation in *E. coli* (17).

SHI-2 locus

The SHI-2 locus encompasses a 30 kB region, including several genes associated with aerobactin iron transport (*iucA-D, iutA*), colicin immunity (*shiD*), and dampening the host inflammatory response (*shiA*) (371; 566). This locus is located 16 bp downstream of another tRNA gene, *selC*, and directly upstream of a disrupted *nlpA* gene. Similar to the SHI-1 insertion site, the region surrounding the SHI-2 locus is also a common insertion site for *E. coli* and *Salmonella* PAIs (371). SHI-2 is not entirely conserved in all *Shigella* strains, however. In the *S. flexneri* 5a strain M90T, the locus downstream of *iutA* is altered: it contains an IS600 element instead of IS2, and lacks several hypothetical ORFs, resulting in a 23.8 kB locus rather than the typical 30 kB (371). In *S. flexneri* serotypes 1a and 2b, *shiD* appears to be inactive, likely due to genetic polymorphisms within this gene (566). Strains of *S. boydii* do not appear to harbor the SHI-2 locus at all (440).

In the microbial pursuit to acquire valuable iron from the host environment, two siderophores are commonly produced by members of the *Escherichia/Shigella* genus: aerobactin and enterobactin (456). All wild type *Shigella* appear to synthesize and transport at least one of these two iron chelators (312). The aerobactin operon, *iuc* (iron uptake chelate), consists of four genes that synthesize this siderophore: *iucA, iucB, iucC,* and *iucD* (35). *iutA* (iron uptake transport), a SHI-2 gene outside the aerobactin operon, encodes the outer membrane receptor for ferric aerobactin (560). In iron-limited environments, aerobactin is released to scavenge free or bound iron, such as transferrin or lactoferrin. The iron-bound aerobactin is then recognized by the IutA receptor and shuttled back into the bacterium (312). The presence of aerobactin provides *Shigella* with an *in vivo* growth advantage when extracellular (i.e., prior to invasion); within the

epithelial cell, however, host hemin or hematin can be directly transported into the cell to serve as the primary iron source, and a siderophore is no longer required (312; 384).

Colicins are molecules produced by enteric bacteria that target and kill neighboring bacteria, a process designed to circumvent competition for valuable resources (93). These toxic peptides bind to specific receptors on a sensitive bacterium, are subsequently translocated via Tol or TonB machinery, and then initiate death in a manner dependent on the colicin; nucleotides and phospholipid bilayers are common targets. To avoid death, bacteria synthesize immunity proteins, which typically either block pore-forming colicins from reaching their target, or bind directly to nuclease colicins to prevent their catalytic activity (93; 269; 574). The SHI-2 locus contains the gene *shiD*, which encodes for immunity against colicins V, lb, and an uncharacterized colicin produced by *S. flexneri* strain 2a SA100, all of which bind to the Cir protein (566). Interestingly, although *shiD* has been demonstrated to produce functional protein, it shares no significant sequence similarity with any known colicin immunity genes, leaving its mechanism of action unknown (371).

shiA, located upstream of the aerobactin operon in SHI-2, plays a role in attenuating *Shigella* inflammation (266). To study the impact of SHI-2 on inflammation, a SHI-2 deletion mutant was constructed in *S. flexneri* 5a strain M90T and rabbit ligated ileal loops were infected with either the resulting mutant or the wild type parent strain. Infection with the mutant resulted in significantly more blunted villi, a hallmark of increased inflammation in intestinal tissue. *shiA* alone was sufficient to repress this hyper-inflammatory phenotype. Conversely, a Δ*shiA* mutant increased the number of apoptotic cells and PMNs within infected villi. Taken together, this evidence suggests

that ShiA plays a role in dampening the immune response, although its mode of action remains unclear; it does not appear to regulate the common mediators of pro-inflammation (266).

Of the remaining proteins encoded by genes in the SHI-2 locus, several share sequence homology to characterized proteins: ShiB to a DNA helicase (95% identity); ShiC to a member of the major facilitator superfamily transporters (56% identity); and ShiF to a tetracycline transporter (34% identity) (242; 566)(BLAST analysis conducted on 01/13/15 (22)). The remaining SHI-2 locus consists of either insertion/transposition elements or novel ORFs with no sequence similarity to characterized genes. There is also a single gene (*int2*) at the beginning of SHI-2 that shares homology with the bacteriophage P4-like integrase, suggesting that this PAI was possibly acquired by bacteriophage integration (566).

SHI-3 locus

The 21 kb SHI-3 locus is found exclusively in *S. boydii*, which lacks the SHI-2 PAI found in *S. flexneri* and *S. sonnei*. The SHI-3 locus encodes a fully functioning aerobactin system that shares 97% nucleotide identity with the SHI-2 aerobactin system; however, it is located between *lysU* and the *pheU* tRNA gene rather than downstream of *selC* (440). Intriguingly, genome comparisons between *S. boydii* and *E. coli* K-12 indicate that the insertion of SHI-3 corresponded with the loss of a 6 kb region that included *cadA*, a known antivirulence gene in *Shigella* (171; 342; 346; 440). It is possible that insertion of the PAI resulted in the immediate deletion of *cadA*, and therefore increased *Shigella* pathogenicity not only through acquisition of a valuable siderophore system but also through loss of an antivirulence gene. However, there is no way to

determine whether or not the *cadA* gene was present directly prior to the insertion event. If the genes in this region were already in decay, the *lysU-pheU* junction may have been an ideal insertion site for SHI-3, as interruptions in regions with essential genes can be deleterious to the organism. SHI-3 also contains numerous insertion/transposition elements and an integrase gene (*int3*), which, like *int2* from SHI-2, is a member of the P4 bacteriophage integrase family.

SRL locus

SRL (*Shigella* resistance locus) is a 66 kb pathogenicity island that carries a functional ferric di-citrate uptake system in addition to multiple antibiotic resistance cassettes (328). Located in the chromosome, the SRL begins 161 bp upstream of *int* and ends at the 3' terminus of *serX*, and contains 59 ORFs. Although not universally present in *Shigella*, 35 out of 55 *Shigella* strains tested by Luck *et al.* identified and linked the SRL genes *tet* and *fec*, suggesting that this PAI is widespread throughout the genus (328). Moreover, the SRL has been identified in strains of each of the four species.

The *fec* iron transport locus contains three operons: *fecI*, *fecR*, and *fecABCDE* (328). *fecI* and *fecR* encode the positive regulatory elements of the system, and the *fecABCDE* operon encodes FecA, the receptor involved in binding ferric di-citrate, as well as structural genes required for the transporter (510; 569). Although the mechanisms involved in ferric di-citrate uptake are still unclear, the structural apparatus for this system consists of a periplasmic protein (FecB), two nonpolar integral membrane proteins (FecC/FecD), and a membrane-bound protein that is thought to bind ATP (FecE) (510). In *Shigella*, the *fec* locus shares 99% nucleotide identity with *E. coli* K-12 genes, and is able to complement an *E. coli* Δ*fec* strain for growth under iron-limited conditions

(328). Furthermore, the *fec* locus is transcribed in *Shigella*; however, this system may be redundant in the presence of other iron uptake systems, as a strain lacking the *fecABCDE* locus shows no growth defect under iron-limiting conditions compared to an isogenic strain carrying the locus. Because the ferric di-citrate uptake system is commonly expressed in commensal *E. coli*, its presence in *Shigella*, like the aerobactin system, may confer a survival advantage within the intestine rather than within the host cell (328).

A considerable number of antibiotic resistance genes also lie on the SRL, including several which encode resistance to streptomycin (*aadA1*), ampicillin (*oxa-1*), chloramphenicol (*cat*), and tetracycline (*tetA*) (328). Many of the remaining ORFs of SRL are homologous to the genes found on the prophages CP4-44, CP4-57, and 933L (328). A number of IS elements, both intact and disrupted, also litter the PAI.

SHI-O loci

Serotyping in *Shigella* is dependent on the O polysaccharide side chain of the cell envelope lipopolysaccharide (LPS) (499). A linear tetrasaccharide repeat forms the backbone of the O side chain in *S. flexneri,* and, in the absence of modifications, is considered the parent serotype Y: -3)-*β*-D-L-Glc*p*Nac-(1-2)-*α*-L-Rha*p*I-(1-2)-*α*-L-Rha*p*II-(1-3)-*α*-L-Rha*p*III-(1- (214). At least three temperate bacteriophages (SfX, SfII, and SfV) are responsible for the glucosylation of different rhamnose residues along this backbone (214; 259; 344). The resulting O-antigen variants produce unique *S. flexneri* serotypes. Such a mechanism is mutually beneficial for both phage and bacteria; certain bacteriophages induce specific O-antigen modifications as a defense to exclude homologous phages from infecting the same bacterial host, and diverse O-antigen

variation can help the bacteria to evade or delay host immune recognition (411; 424). Collectively, these bacteriophages form the SHI-O loci.

The SfX bacteriophage encodes three O-antigen modification genes: *gtrX*, a glucosyltransferase; *gtrA*, a small hydrophobic protein of unknown function; and *gtrB*, a bactoprenol glucosyltransferase (214; 564). First, GtrB transfers a glycosyl group from UDP glucose to the lipid carrier undecaprenyl phosphate (UndP) (214) Although the exact function of GtrA is unclear, it is thought to play a role in the subsequent translocation of lipid-linked glucose across the cytoplasmic membrane. Finally, GtrX attaches the glucosyl group to the first rhamnose residue of the growing O side chain, altering the *S. flexneri* serotype from Y to X.

The remaining two bacteriophages, SfII and SfV, share similar gene organization to SfX in regards to their respective O-antigen modification genes. In SfII, *orf2* and *bgt* share high homology to *gtrA* and *gtrB* respectively, and likely produce proteins of similar functions (344). The amino acid sequence of GtrII, the glucosyltransferase of SfII, is divergent from GtrX, however, likely due to differences in target specificity. GtrII mediates the attachment of a glycosyl group to the third rhamnose unit of the O side chain, producing *S. flexneri* serotype II. Three serotype conversion genes (*orf4*, *orf5*, and *gtrV*) are likewise present on the temperate bacteriophage SfV (259). Like both SfX and SfII, *orf4* and *orf5* encode proteins with high identity to GtrA and GtrB; only *gtrV*, the putative glucosyltransferase, is divergent. GtrV catalyzes the transfer of a glucosyl group to the second rhamnose unit, resulting in *S. flexneri* serotype V.

DRUG RESISTANCE

Shigella, like many bacterial pathogens, has become more resistant to antimicrobials over the past few decades. The rise of multidrug-resistant (MDR) *Shigella* strains poses critical health concerns for future treatment of infected individuals, particularly in the absence of an effective vaccine. While the majority of MDR *Shigella* outbreaks in the last few years have occurred in southern Asia (India, Bangladesh, and China), reports have also arisen of MDR outbreaks in South America (Chile) and the Middle East (Iran) (55; 418; 531; 545; 554; 604; 605).

One possible explanation for the rapid worldwide distribution of MDR *Shigella* strains is a substantial flux in the epidemiological landscape of this pathogen over the course of the last century. International travelers are an underappreciated and significant factor for dissemination of *Shigella* species across the globe (27; 246). Travelers may introduce novel MDR strains to naïve populations; these strains may then become endemic to the region, particularly if they are more resistant to eradication by antibiotics than native circulating strains. Asymptomatic carriage of *Shigella* isolates may also account for persistence of these MDR strains, as an environmental reservoir for the bacteria has not yet been identified (75). In developing nations, which share the largest burden of *Shigella* infections, antibiotic use is often unregulated, a circumstance that can lead to the misuse and overuse of antibiotics (230). Asymptomatic carriers of *Shigella* strains may be exposed to broad spectrum antibiotic drug treatment meant to target other infections, which could provide ample selective pressure for keeping these MDR strains circulating in the population.

Traditional first-line antibiotic treatment for *Shigella* included ampicillin, trimethoprim-sulfamethoxazole (co-trimoxazole), and nalidixic acid (587). However,

many of these first-line drugs have become ineffective and are no longer recommended for therapy due to widespread resistance. Ciprofloxacin has become the antibiotic of choice for MDR strains; however, rising resistance to this antimicrobial has also been reported (179). Second-line antibiotics include other fluoroquinolones, pivmecillinam (amdinocillin pivoxil) and ceftriaxone (587). Azithromycin can also be used to treat adults and children (587).

The mechanisms for antimicrobial resistance in *Shigella* have been well characterized. Ampicillin resistance is mediated by beta-lacatamases, which are encoded by the *bla* genes, such as bla_{TEM}, bla_{OXA}, and bla_{SHV} in *Shigella* (11; 545). In *E. coli*, acquisition of the alternative dihydropteroate synthase (DHPS) genes (*sul1*, *sul2*, or *sul3*) results in trimethoprim-sulfamethoxazole resistance (423). All three of these genes have been identified in isolates of *Shigella* (101; 419). Finally, the mechanism for quinolone resistance (ciprofloxacin and nalidixic acid) involves the accumulation of one or more specific mutations in the chromosomal quinolone resistance determining regions (QRDR) or the plasmid-mediated quinolone resistance determining regions (PMQR). Target genes include the DNA gyrase (*gyrA*), topoisomerase IV (*parC*), aminoglycoside acetyltransferase (*aac(6')-Ib-cr*), an efflux pump (*qepA*), and a pentapeptide-repeat family protein (*qnr*) (534). The subsequent dissemination of mobile genetic elements, such as plasmids, transposons, and integrons, are common routes for the rapid spread of antimicrobial resistance genes among circulating strains (37; 198; 336).

CONCLUDING REMARKS

The ability of *Shigella* to survive the hostile conditions of the gastrointestinal tract, invade the colonic epithelium, and manipulate the immune system of the human

host requires a full complement of diverse virulence factors, including an impressive array of T3SS effectors and, in certain strains, PAI-associated factors, Shiga toxin, and/or antibiotic resistance genes. Although the virulence phenotypes of *Shigella* have been extensively studied thus far, much work remains to fully expose the mechanics of this organism. The facets of *Shigella* pathogenesis that remain the least understood include, but are not limited to, the T3SS-mediated manipulation of the host immune response; the complete characterization of the *Shigella* PAIs; and the determination of specific mechanisms involved in T3SS effector activities. Future investigations of these topics will continue to reveal the complex interactions of this versatile pathogen with its host. This work is critical to not only advance our knowledge of *Shigella* virulence, but also to develop new treatments or preventions against this pathogen. To date, no effective vaccine has been developed to protect susceptible populations against *Shigella*, and the number of people who contract shigellosis annually is staggering. To complicate matters, the emergence of MDR strains hampers the effective treatment of patients with bacillary dysentery.

CHAPTER 4: *SHIGELLA* AND ANTIVIRULENCE: THE DARK SIDE OF BACTERIAL EVOLUTION (63)

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ABSTRACT

The acquisition of novel virulence factors and subsequent remodeling of bacteria systems to incorporate these factors are equally important events in pathogen evolution. Genes that are inactivated or lost from an emerging pathogen's genome due to incompatibility with new virulence factors are known as antivirulence genes (AVGs). AVGs were first described in *Shigella*, a human-specific gastrointestinal pathogen that evolved from ancestral *Escherichia coli*. Although AVGs have been described in a variety of bacterial pathogens, including *Salmonella*, *Yersinia*, *Burkholderia*, and *Francisella*, *Shigella* remains perhaps the best model organism for the study of antivirulence due to the vast extent of literature on not only *Shigella*, but also *E. coli*, which retains the functional AVGs that have been inactivated or lost in *Shigella* strains. Currently, there are five documented AVGs in *Shigella*: *cadA*, *nadA/nadB*, *speG*, and *ompT*. In this chapter, we will discuss the discoveries of these AVGs, their effects on virulence, and the events that led to their inactivation or loss in *Shigella*. The development of novel therapeutics and vaccines, an improved understanding of virulence mechanisms, and a greater insight into pathogen evolution are just some of the benefits that may arise from exploration of AVGs in bacterial pathogens.

INTRODUCTION

The acquisition of virulence genes via horizontal gene transfer can be crucial to the ability of bacteria to colonize novel niches. Extrachromosomal elements, such as plasmids or bacteriophages, or conjugation between strains to share genes or pathogenicity islands, can all contribute to the incorporation of new DNA into the

bacterial genome (212; 314). As a result, a pathogen may arise from a formerly harmless commensal, or a pathogen may shift from one specialized niche to another.

Given the complex nature of bacterial gene/protein regulation and function, newly-acquired virulence factors do not always integrate smoothly into established bacterial systems (339; 505). Any functional conflict between these new virulence genes and the remainder of the bacterial genome may therefore compromise bacterial fitness. Moreover, colonization of a novel pathogenic niche can add new selective pressures for the bacterium to overcome. If the resulting fitness cost to the bacteria is too extreme, variant strains that avoid or bypass such functional conflict in the new niche will be selected for.

If a bacterium acquires virulence genes that conflict with an existing bacterial network, two outcomes are possible. In the first scenario, a bacterium may simply lose or inactivate the newly-acquired virulence genes. Bacteria that have not spontaneously lost or inactivated these genes could otherwise risk predation or being out-competed by other bacterial strains/species, and will therefore not survive to pass their own genes to the next generation. This concept is a part of antagonistic pleiotropy, which states that any gene (or gene system) whose fitness costs outweigh the benefits in a certain environment will be selected against (584).

Conversely, if the newly-acquired virulence genes confer significant fitness benefits to the bacterium, they will be more likely to be selected for, and existing genes may be subject to a 'fine-tuning' process to optimally accommodate the new genes. In this second scenario, the bacterium will maintain the newly acquired virulence genes and modify the existing genome through gene loss (gene inactivation/deletion) or positive

selection for compensatory mutations in existing genes, a process known as pathoadaptive evolution (505).

Optimization of bacterial virulence through pathoadaptive gene loss is a specialized form of pathoadaptive evolution known as antivirulence (339; 342). An antivirulence gene (AVG) is a gene whose expression in a pathogen is incompatible with the virulence of that pathogen (62). Consequently, virulence may be enhanced through gene inactivation or partial/full deletions, otherwise known as 'black holes.'

Shigella was the first organism in which AVGs were described. In the past two decades, however, AVGs in other bacterial pathogens have been identified (for a complete review, see (62)). To date, there are over 10 known AVGs in at least four other bacterial pathogens, including *Yersinia*, *Salmonella*, *Burkholderia*, and *Francisella* (154; 155; 157; 223; 365; 366; 450; 522). While this chapter focuses exclusively on AVGs of *Shigella*, studies describing AVGs in other bacterial species are certainly worthy of note and could be a gateway to discoveries of novel AVGs in *Shigella* and other pathogens. These studies also represent the very broad range of molecular functions that must be attenuated in these pathogens to permit virulence, including inactivation or loss of genes involved in biofilm synthesis, metabolism, lipopolysaccharide modification, and host vasoconstriction. This is an excellent reminder that the dynamics of bacterial fitness and pathogenicity are complex, and the breadth of AVGs mirrors that complexity.

Finally, the importance of discovering and describing these AVGs goes beyond merely satisfying academic curiosity and obtaining a better understanding of how these pathogens evolve. There are practical applications. Identification of AVGs may provide a basis for the development of novel therapeutics in the form of inhibitor compounds

specific for pathogen virulence factors. With the rise of antimicrobial resistant pathogens, including multi-drug resistant *Shigella* isolates, the development of novel therapeutics is more important than ever (11; 213; 598). New drugs with a high specificity for pathogenic targets are desirable in that they circumvent disruption of commensal bacteria or the host's own cells. AVG study may also provide beneficial insight into bacterial pathogenicity by helping to uncover novel virulence factors or offer clues to the mechanisms behind already known virulence factors. Lastly, AVGs may aid in vaccine development, which has been experimentally demonstrated with a *Yersinia* AVG, *lxpL* (365).

THE EVOLUTIONARY HISTORY OF *E. COLI* AND *SHIGELLA*

Despite boasting a distinct genus classification, *Shigella* is taxonomically grouped as a unique pathovar within *E. coli* (308; 438). In fact, bioinformatics-based evidence indicates that *Shigella* recently branched from ancestral *E. coli* strains between 35,000 to 270,000 years ago (438). Acquisition of a large virulence plasmid, the defining evolutionary event for *Shigella* evolution, likely occurred up to seven independent times. This suggests that modern *Shigella* strains are descendants of not one but several different ancestral *E. coli* lineages.

In the process of evolution, *Shigella* strains rose to colonize a very unique, specific niche compared to the broader natural habitat that modern commensal *E. coli* isolates are known to populate, evolving from extracellular ancestors into intracellular pathogens capable of colonizing human intestinal epithelial cells (Figure 2) (438). This ability was attained primarily via lateral transfer of a large 220 kb virulence plasmid that houses genes that encode for a type three secretion system (T3SS) and various secreted

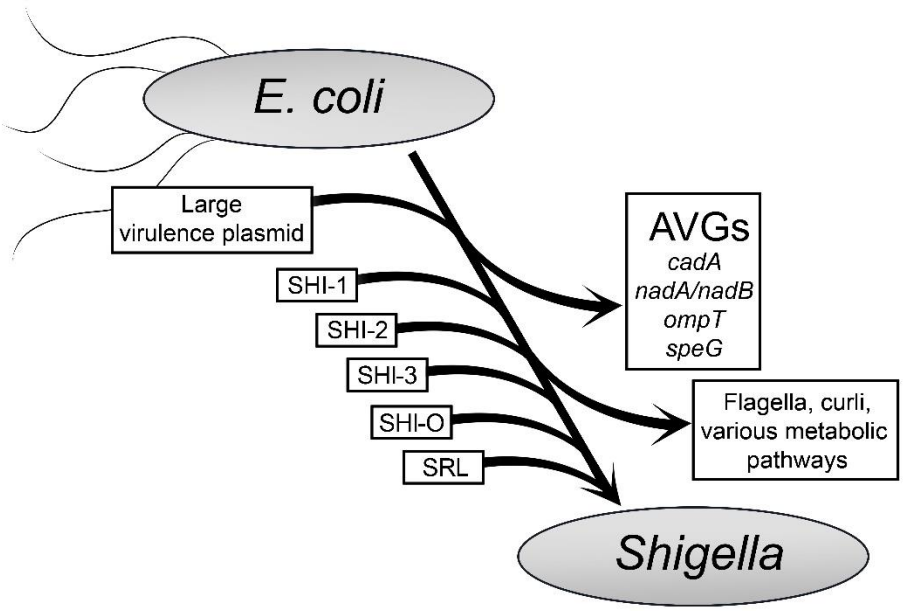

Figure 2. Evolution of *Shigella* from *E. coli*.
Although *Shigella* acquired several characteristic virulence factors, including a large virulence plasmid and four pathogenicity islands (SHI-1, SHI-2, SHI-3, SHI-O, and SRL), it also lost AVGs (*cadA*, *nadA/nadB*, *ompT*, and *speG*) in addition to flagella, curli, and various metabolic pathways.

effectors (487). Furthermore, several distinct *Shigella* pathogenicity islands (SHI-1, SHI-2, SHI-3, SHI-O, and SRL) were also transferred to the chromosome, although their presence varies between strains (421). These genes encode virulence factors including an enterotoxin, an immunoglobulin A-like cytotoxic protease, a serine protease, a T-cell signaling suppressor, and molecular systems for aerobactin iron acquisition, O antigen modification, and ferric di-citrate uptake, in addition to antimicrobial resistance determinants (487). Various smaller plasmids may be present depending on the *Shigella* strain, and have been known to carry additional antibiotic resistance genes that may deliver an edge in bacterial survival within the human gut (101; 231). Lastly, *S. dysenteriae* serotype 1 strains carry the *stxA/stxB* genes encoding Shiga toxin, an A_1B_5 toxin capable of cleaving a single adenine residue from the 28S rRNA of the eukaryotic host cell to inhibit host protein synthesis (537).

Although modern *E. coli/Shigella* strains share a common core genome, there are obvious genetic and phenotypic differences that distinguish the two organisms. During adaptation to their highly-specific host niche, *Shigella* underwent substantial reductive evolution, and their genomes reveal a high frequency of chromosomal pseudogenization when compared to strains of non-pathogenic *E. coli* (597). Shortly following gain of the major virulence plasmid, *Shigella* likely started inactivating AVGs inhibitory to invasion into or survival within the eukaryotic host cell (339). Probably more gradually, *Shigella* strains also began accumulating inactivating mutations in genes unnecessary for survival in the new niche (in other words, neutral genes which confer neither a positive nor negative selective advantage within human intestinal epithelial cells) (367). On average, there are over 200 pseudogenes per strain in the *Shigella* species, including many genes

involved in carbon utilization, cell motility, and transporter/membrane proteins (170; 597). In one study, 85 genes were found to be commonly decayed across five sequenced *Shigella* strains, with a rather staggering 1,456 genes inactivated in at least one strain, suggesting that the capacity for genome reduction is high (170). Insertion elements are a common form of gene disruption, and *Shigella* strains are rife with them, with anywhere between 300-700 copies of insertion sequence elements per strain (597).

It is impossible to discuss *Shigella* in the context of antivirulence without also mentioning enteroinvasive *E. coli* (EIEC), another human-specific pathogen that causes diarrhea in humans. EIEC share many genetic and phenotypic similarities with the shigellae, and are thought to be more recently emerged pathogens that harbor the same T3SS-encoding large virulence plasmid, which allows them to inhabit the same intracellular niche (308). Although EIEC are not considered to be ancestral strains of the modern *Shigella* isolates, they may be following a parallel evolutionary pathway (308).

Like *Shigella*, EIEC strains are characteristically nonmotile and unable to ferment lactose, although these phenotypes are not as universal across the EIEC strains (498). In contrast to *Shigella*, however, EIEC strains share more similarities with commensal *E. coli* isolates, including the ability to ferment xylose and produce gas from glucose (498). Although we will not discuss EIEC in this chapter, it is important to note that many of the phenotypes resulting from loss of AVG in *Shigella*, including the inability to decarboxylate lysine, are also characteristic of EIEC strains (92; 498).

SHIGELLA, THE ENVIRONMENT, AND THE HUMAN HOST

As an intracellular, host-specific pathogen that has undergone a substantial amount of reductive evolution, *Shigella* is probably less fit within the environment

compared to environmental or commensal *E. coli.* While the majority of *E. coli* isolates are prototrophic and capable of surviving in a wide variety of niches, *Shigella* isolates are universally auxotrophic for nicotinic acid, and certain strains (particularly *S. dysenteriae* isolates) also require supplementation with tryptophan or methionine (12). Furthermore, *Shigella* strains universally lack a flagellar system and membrane-associated adhesions such as curli (19; 467; 543). These bacterial appendages may confer environmental survival advantages to *E. coli* strains through enhanced nutrient access or biofilm formation, respectively (332; 577). The capacity of *Shigella* to remain virulent following prolonged periods outside its host is thought to be limited, which may be due to inactivation of genes found on the large virulence plasmid or even loss of the virulence plasmid itself (164; 442). Lastly, maintenance of such a large plasmid can be an energy drain on the bacterium, and plasmid loss may be selected for in *Shigella* isolates outside the host niche, as has been demonstrated during periods of nutrient starvation in *E. coli (84)*.

Conversely, *Shigella* thrives in the replicative niche of the human epithelial cell cytoplasm (487). The plasmid-encoded T3SS is an essential requirement for initial entry and subsequent survival of *Shigella* within the human intestinal epithelium. It has diverse functions in epithelial cell invasion, bacterial replication, and cell-to-cell spread, and it also regulates the host inflammatory response necessary for optimal bacterial survival (487). The T3SS of *Shigella* likely plays a role in not only virulence, but overall bacterial fitness of the pathogen within the human host.

Although we do not yet know if genes of the large virulence plasmid play any particular role(s) while *Shigella* is in the environment, the necessity of the T3SS within

the human host is well-documented, and attenuation of the T3SS in this niche by any AVGs would be a significant impediment to bacterial fitness.

THE KNOWN AVGS OF *SHIGELLA*

cadA

One of the primary factors used to distinguish strains of *Shigella* from *E. coli* in the clinical microbiology laboratory is the inability of the *Shigella* strains to decarboxylate lysine into the polyamine cadaverine (Figure 3). In *E. coli* strains, the *cadA* gene encodes for an acid-inducible lysine decarboxylase, which is transcribed in an operon with *cadB*, a gene that encodes for a lysine/cadaverine antiporter. This operon is induced by the transcriptional activator CadC, a member of the ToxR-like family of proteins, which binds the *cadBA* operon in the presence of exogenous lysine and an acidic external pH (305).

Over 90% of *E. coli* strains encode a functional lysine decarboxylase (143). In contrast, *Shigella* strains have inactivated or even completely lost this function through a variety of different mutations, including partial or full deletions of the *cadBA* operon, nonsense mutations, missense mutations, and the disruption of open reading frames by insertion elements, loci from other chromosomal regions, or even phage genomes (122). This vast mutational diversity suggests that negative selective pressure against this locus was strong in the *Shigella* ancestor strains, and inactivation of this locus was required for bacterial fitness in the new niche. Convergent evolution is a common theme among the shigellae with respect AVG loss. Like *Shigella*, EIEC strains are negative for lysine decarboxylase activity and do not produce cadaverine. Unlike *Shigella*, however, some

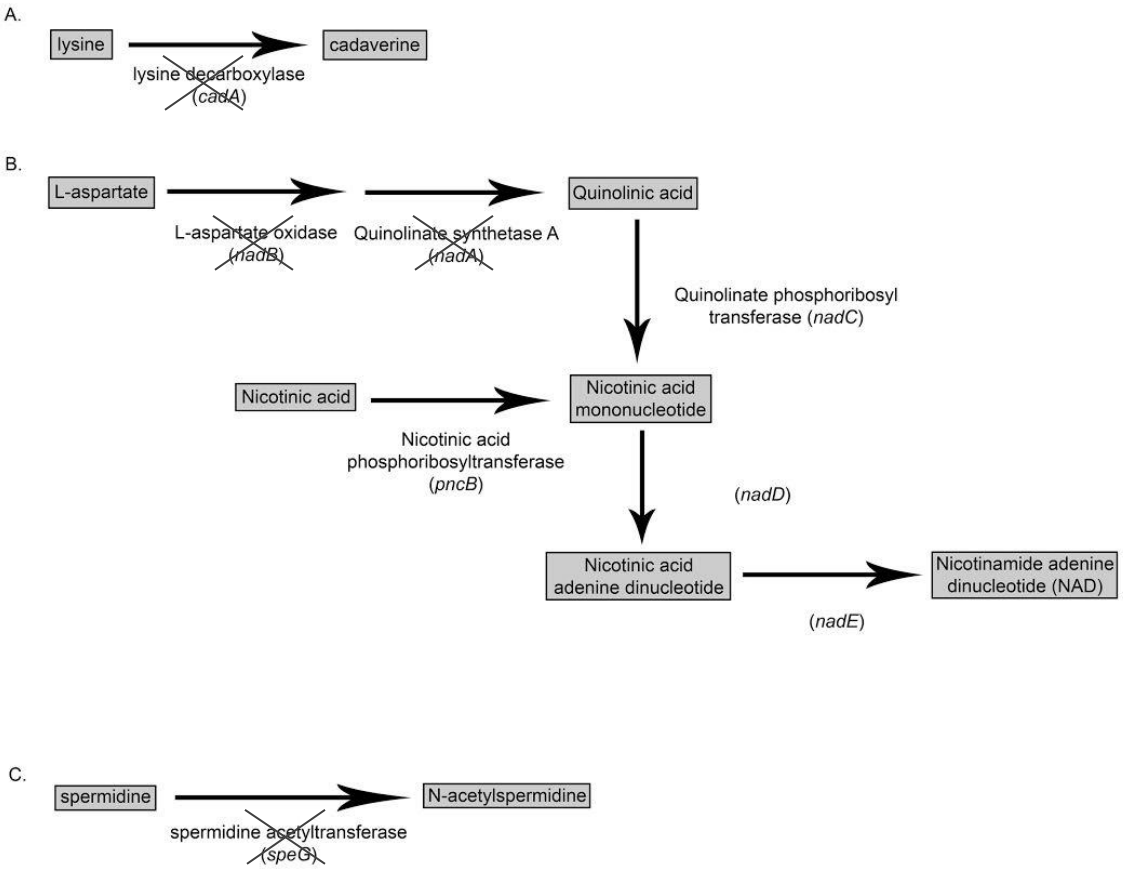

Figure 3. Metabolic pathways lost or interrupted in *Shigella*.
A.) Lysine decarboxylation. B.) Biosynthetic and salvage NAD pathways. C.) Spermidine metabolism. Genes that have been inactivated or lost in *Shigella* are crossed out.

EIEC strains have maintained a functional *cadA* gene (92). The transcriptional activator *cadC* has instead been inactivated in most EIEC strains through open reading frame disruption with different insertion elements. Loss of CadC activity prevents expression of the *cadBA* operon and production of the lysine decarboxylase. In the remaining EIEC strains, deletion of the *cadBA* operon has led to the lysine decarboxylase negative phenotype.

When a functional lysine decarboxylase system is restored in a wild type *Shigella* strain in *trans*, several different *Shigella* virulence phenotypes are attenuated (Figure 4) (171; 342; 346). The ShET1 and ShET2 enterotoxins, located on the bacterial chromosome and the large virulence plasmid, respectively, are thought to contribute to the initial phase of watery diarrhea during dysentery (165). In the presence of a functional *cadA* from an *E. coli* K-12 strain, the ability of an *S. flexneri* strain to induce fluid secretion is blocked (342). Secondly, bacterial phagosome escape, an essential step for subsequent *Shigella* replication in the host cytoplasm, is prevented during *cadA* expression (171). Finally, polymorphonuclear leukocyte (PMN) influx, an inflammatory event that may be essential for bacterial access to the preferred basolateral side of the intestinal epithelium, is attenuated (346). Cadaverine, the end product of the lysine decarboxylase reaction, is responsible for attenuation of these phenotypes, as addition of a physiologically relevant concentration of cadaverine to wild type *Shigella* also inhibits virulence.

A mechanism for *cadA*/cadaverine inhibition of *Shigella* pathogenesis has not yet been elucidated. Intriguingly, the *Shigella* virulence phenotypes inhibited may not even be linked, which could suggest that cadaverine may play multiple roles in the attenuation

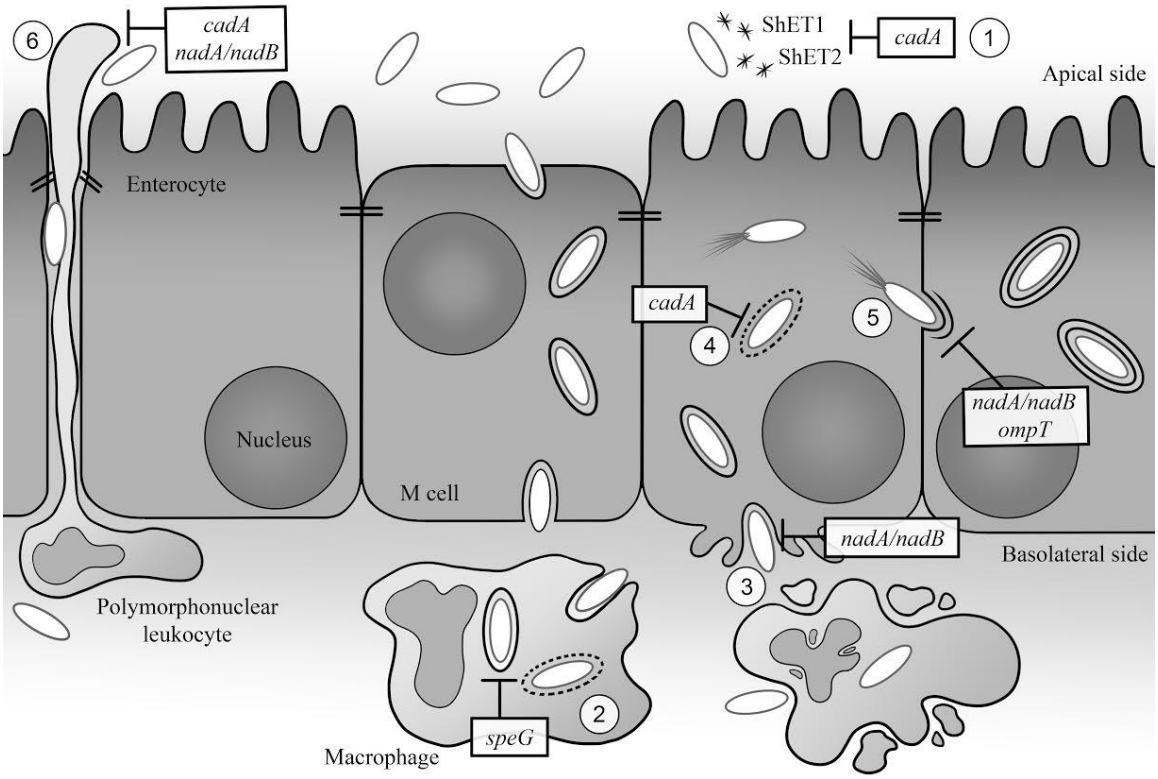

Figure 4. Inhibition of pathogenesis in *Shigella* by AVGs.
During *Shigella* invasion into the human intestinal epithelium, AVGs inhibit various pathogenesis phenotypes. Cadaverine, the product of the lysine decarboxylase reaction, inhibits ShET1/ShET2 enterotoxin activity (1), phagosome escape (4), and PMN transepithelial migration (6). Quinolinic acid, the product of reactions encoded by the *nadA/nadB* genes, inhibits *Shigella* invasion (3), intracellular spread (5), and PMN transepithelial migration (6). Inactivation of the spermidine acetyltransferase *speG* results in the accumulation of spermidine within the phagosome and supports bacterial survival in the macrophage (2). Lastly, the outer membrane protease OmpT cleaves bacterial IcsA, ultimately attenuating actin tail polymerization and inhibiting cell-to-cell spread (5).

of *Shigella* pathogenesis, possibly even protecting the host cell prior to *Shigella* exposure. Polyamines such as cadaverine can play a wide variety of physiological roles in viral, bacterial, and mammalian cells, primarily due to their cationic nature. The net positive charge of the polyamine is distributed over the length of the molecule rather than at a single point, allowing polyamines to bind to or serve as bridges between negatively-charged molecules such as DNA or RNA (496). Most research thus far has focused on polyamine activity in plants and mammals, but in bacterial pathogens, polyamines have been demonstrated to influence microbial carcinogenesis, macrophage apoptosis, biofilm formation, siderophore biosynthesis, and the oxidative stress response (496).

As a curious side note, the *Shigella*/EIEC strains are not the only members of the *E. coli* pathovar to lose function of *cadA*. Torres and colleagues noted that many enterohemorrhagic *E. coli* (EHEC) and Shiga Toxin-producing *E. coli* (STEC) strains also lack a functional lysine decarboxylase gene (546; 562). When *cadA* is expressed in these strains, bacterial adherence to host cells is significantly decreased. This phenotype is likely due to a corresponding decrease in the expression of outer membrane adhesions such as intimin, although it is not yet known through what mechanism this reduction occurs. In contrast to *Shigella*, the end product cadaverine does not appear to play a role in the intimin phenotype. However, as the inactivation or loss of *cadA* in these strains is not universal, *cadA* is not yet considered an AVG in these organisms.

nadA*/*nadB

Another feature that has long been known to distinguish *Shigella* strains from modern *E. coli* isolates is the almost universal inability of *Shigella* to grow on minimal salts media (12). 98% of clinical *Shigella* isolates are auxotrophic for nicotinic acid, and

strains of *S. dysenteriae* are also auxotrophic for tryptophan and methionine (12). Although auxotrophy does exist in *E. coli*, many strains are prototrophic, capable of growing without supplementation (483). To determine whether or not these auxotrophic phenotypes are indicative of AVG loss, the inability of *Shigella* to synthesize nicotinic acid was studied (434).

The biosynthetic and salvage pathways for NAD in *E. coli* have been well-described (Figure 3) (192). The enzymes L-aspartate oxidase (encoded by *nadB*) and quinolinate synthetase A (encoded by *nadA*) form a complex that, in *E. coli*, converts L-aspartate to quinolinic acid. Quinolinic acid is then converted to nicotinic acid mononucleotide, one of the precursors of nicotinamide adenine dinucleotide (NAD). In the salvage pathway, bacteria can take up exogenous nicotinic acid, which is then converted to nicotinic acid mononucleotide by nicotinate phosphoribosyltransferase (encoded by *pncB*).

In a study of 12 *Shigella* strains, changes were revealed in the sequences of *nadA*, *nadB*, or both genes within all strains compared to the functional *nadA*/*nadB* sequences in an *E. coli* K-12 strain (433). Insertion element interruptions of open reading frames, point mutations, and frameshift mutations may have contributed to the nicotinic acid auxotrophy phenotype in these strains. Two EIEC isolates, known nicotinic acid auxotrophs, also demonstrated insertion element disruptions in the open reading frame of *nadB* and several point mutations within *nadA*. However, the *pncB* gene encodes for a functional transferase in *Shigella*, permitting conversion of exogenous nicotinic acid to nicotinic acid mononucleotide. By utilizing this salvage pathway, the pathogen can

completely bypass the *nadA*/*nadB* biosynthetic pathway to satisfy its requirements for NAD (194).

When a wild type *Shigella* strain is exposed to quinolinic acid, several virulence phenotypes are inhibited, including cell-to-cell spread, host cell invasion, and *Shigella*-induced transepithelial migration of PMNs (Figure 4) (434). Similar to cadaverine, there is currently no known mechanism of action for quinolinic acid inhibition of *Shigella* virulence.

Intriguingly, most *Shigella* species appear to retain a functional quinolinate phosphoribosyl transferase (encoded by *nadC*), which can convert quinolinic acid to nicotinic acid mononucleotide. Presumably, in the absence of quinolinic acid biosynthesis, a functional *nadC* may be required to deal with any exogenous quinolinic acid the bacterium encounters, neutralizing this potentially inhibitory small molecule by metabolizing it into a useful form for the bacterium (434).

speG

An important role for the *Shigella* T3SS is to promote bacterial survival in and eventual escape from the human macrophage. During infection, *Shigella* bacteria transcytose through M cells into the Peyer's patches of the large intestine, where they are ingested by resident macrophages (476). The bacteria quickly escape the phagosome and induce the macrophage to undergo pyroptosis, a specialized form of proinflammatory cell death characterized by caspase-1 activation (524). This leaves the bacteria free to invade the intestinal epithelium from the preferential basolateral side (373). To infect its host and effectively replicate, *Shigella* must not only induce pyroptosis in the macrophage but also

survive the hostile conditions of the macrophage phagosome, which include reactive oxygen species such as hydrogen peroxide and nitric oxide (503).

speG encodes for spermidine acetyltransferase, an enzyme which produces N-acetylspermidine through transfer of an acetyl group onto the polyamine spermidine (Figure 3). In *Shigella* strains, *speG* has been lost or inactivated via different deletions, non-synonymous missense mutations, and disruptions of the open reading frame by insertion elements (39). Interestingly, the inactivation or loss of *speG* in *Shigella* is not to prevent production of an inhibitory end product, but to promote buildup of the precursor molecule spermidine (39). Spermidine concentrations in *Shigella* strains, which lack a functional *speG*, are roughly three-fold higher than in *E. coli* isolates, which express *speG*. When a functional *speG* is expressed in *Shigella*, bacteria are less able to withstand the effects of oxidative stress as measured by their survival in the presence of hydrogen peroxide. The expression of a functional *speG* also significantly reduces the ability of *Shigella* to survive within murine macrophages, suggesting that *speG* expression increases *Shigella* sensitivity to the oxidative stress response within the macrophage (Figure 4).

Spermidine, like cadaverine, is one of four major polyamines (putrescine, cadaverine, spermidine, and spermine) found in bacterial cells (496). Spermidine has been credited with a variety of roles in bacteria, including regulation of gene expression, porin conformation regulation, and free radical scavanging (496). Although the mechanism of action for *speG* antivirulence is not yet fully elucidated, expression of *katG*, a gene encoding hydroperoxidase I, is significantly repressed in the presence of functional *speG*. KatG, part of the bacterial oxidative stress defense system, protects

against oxidative stress by converting hydrogen peroxide to water and oxygen, and is transcriptionally regulated by the polyamines putrescine and spermidine in *E. coli* (281). Therefore, loss of the AVG *speG* appears to increase spermidine accumulation, which in turn induces expression of *katG*, ultimately inhibiting macrophage killing of *Shigella* (39).

ompT

One of the hallmarks of *Shigella* pathogenesis is the ability of the bacteria to spread from one epithelial cell to another by hijacking host actin filaments (145). IcsA (also known as VirG), induces actin-based motility by binding the host N-WASP protein, which in turn interacts with host F-actin and functions in attachment of the actin tail to the bacterium (145).

ompT encodes for an outer membrane protease that cleaves IcsA, effectively attenuating cell-to-cell spread in *Shigella* (Figure 4) (381). However, not all modern *E. coli* strains carry the *ompT* gene, which makes classification of *ompT* as an AVG difficult. In *E. coli*, *ompT* sits in the midst of a DLP12 cryptic lambdoid-like prophage downstream of the *envY* gene. In a survey of 144 strains in the EcoCyc database (http://ecocyc.org/), 25% of *E. coli* strains do not carry the prophage or *ompT*, and none of the modern *Shigella* isolates studied even carry remnants of the prophage in this position (62; 291). Therefore, we do not know if the *Shigella* ancestral lineages ever carried *ompT*. For that reason, *ompT* is the most ambiguous gene on the current list of *Shigella* AVGs, although at the present time, we include *ompT* as a *Shigella* AVG.

IDENTIFICATION OF *SHIGELLA* AVGS

Identification of novel AVGs remains a slow and rather arduous process. Bioinformatics analyses, while improving, still present significant challenges. Genome annotation for pseudogenes is mediocre at best, and even obvious pseudogenes, such as partial/full gene deletions and premature nonsense mutations, may not be annotated correctly. Furthermore, a pseudogene that results from a non-synonymous missense mutation can usually only be classified as non-functional through experimental characterization. An ideal bioinformatics program would predict structural changes resulting from such missense mutations and determine the resulting consequence on protein functionality. Continuous improvement of bioinformatics platforms is critical, therefore, to enhance the identification and study of AVGs.

If a putative AVG target is identified, it must then be investigated in the context of the entire network to which it belongs. Many genes are part of larger biosynthetic pathways or bacterial systems, and when one gene is inactivated, the entire pathway or system may be nonfunctional or interrupted. This is especially true for organisms such as *Shigella*, which may still be in the process of genome pseudogenization of unnecessary genes. Furthermore, the same gene within a pathway or system is not always preferentially targeted for inactivation in all strains, suggesting that similar selective pressures may have led to convergent evolution. As an example, although *nadA* and/or *nadB* is always inactivated in *Shigella*, some strains appear to maintain one of the two genes in a functional state (433). Loss of one of these two enzymes is sufficient to prevent quinolinic acid from being produced. If only *nadA* had been examined across all *Shigella* strains, this particular AVG locus may have been missed. The decaying flagellar system is another an excellent example (19; 543). In some isolates, certain flagellar

genes, such as *fliC*, are able to fully complement *E. coli* mutants, demonstrating functionality of these genes (544). However, other genes within the flagellar system (such as the *flhDC* operon) have been inactivated or lost, and *Shigella* strains demonstrate a universally non-motile phenotype (19).

Even when pseudogenes are properly identified, the scientist must then determine whether a gene was inactivated or lost because it was unnecessary to bacterial survival in the new niche, or because it was an AVG that inhibited bacterial virulence. Currently, this distinction is impossible to make without re-introduction of the putative AVG into the bacterium and subsequent characterization of virulence phenotypes in the presence or absence of the AVG.

CONCLUDING REMARKS

Following lateral acquisition of novel virulence factors, emerging pathogens must optimally incorporate these genes into their existing genome. This is accomplished through loss of genes inhibitory to virulence in the new niche, otherwise known as antivirulence genes (AVGs). *Shigella* is an excellent model organism for the study of AVGs because of the high incidence of pseudogenes present in *Shigella* strains the wealth of knowledge available for both the pathogenicity and basic biology of *Shigella* and its extant cousin *E. coli*, and the accessibility of an increasing number of sequenced *Shigella* genomes. The discovery of AVGs in *Shigella* and other bacterial pathogens may help pave the way for the creation of highly pathogen-specific therapeutics, the unearthing of novel virulence targets, and the development of new vaccines.

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CHAPTER 5: THE *S*MIP: A NOVEL TOOL FOR STUDYING THE CONTRIBUTIONS OF *SHIGELLA* T3SS GENES TO VIRULENCE (64)

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The data presented in this chapter are preliminary and will be submitted for publication upon completion of analysis.

K. A. Bliven is responsible for the work presented in this chapter, with the following exceptions: Y. Anriany and A. Prunier constructed the *S*mip/*S*mip-*icsA*, and M. Rosselin did the fluorescence microscopy experiment. K. A. Bliven completed all other experiments and wrote the chapter. All authors assisted with experimental design, and K. A. Bliven, M. Rosselin, and A. T. Maurelli analyzed the data.

Note: The figures, tables, and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

Infections with *Shigella*, a Gram-negative, rod-shaped bacterium, can result in shigellosis, a human-specific enteric disease spread via the fecal-oral route. The primary virulence factor for this pathogen is a type three secretion system (T3SS) encoded on a large 220 kb virulence plasmid present in all *Shigella* species. Host cell invasion, cell-to-cell spread, and immune system manipulation are dependent on assembly of the T3SS needle and secretion of roughly 20 effectors. Although many effectors have been characterized, functional redundancy between several of the remaining T3SS effectors have made full characterization of this system difficult. To address this problem, a reductionist strategy was employed. The minimal T3SS genes necessary and sufficient for entry into the cell were cloned into a fosmid backbone along with the T3SS transcriptional activators, *virF* and *virB*. The resulting construct, termed the *S*higella minimally invasive plasmid (*S*mip), was introduced into a plasmid-cured strain of *S. flexneri* (BS103) and common *Shigella* virulence phenotypes (invasion, cell-to-cell spread, and T3SS secretion) were studied. Because the *S*mip lacks the T3SS effectors required for post-entry events, these effectors can be added back to the strain alone or in combination to study their respective effects on virulence. As a proof of principle for the utility of the *S*mip, *icsA* and the T3SS effector *virA* were expressed in the BS103/*S*mip strain to demonstrate their contributions to cell-to-cell spread. In addition to effector characterization, this novel tool may assist in the identification of new *Shigella* antivirulence genes or development of an effective vaccine.

INTRODUCTION

Shigellosis is a potentially devastating enteric disease that can elicit fever, abdominal cramps, and bloody diarrhea. Roughly half a million episodes occur yearly in the United States alone, while globally, 164.7 million people suffer from this disease (98; 303). Over one million infections result in death; children under the age of five in developed nations are the most susceptible cohort (303). The causative agent of this disease is the Gram-negative, rod-shaped, human-specific bacterium, *Shigella.* As few as 10-100 organisms, spread via the fecal-oral route, are required to cause disease (140). Currently, there is no protective vaccine available.

The pathogenic *Shigella* species evolved directly from commensal *Escherichia coli* strains around 35,000-270,000 years ago following acquisition of a large 220 kb virulence plasmid, termed the pINV (341; 438; 477). The pINV encodes the pathogen's primary virulence factor, a temperature-dependent type three secretion system (T3SS) required for *Shigella* invasion into the human intestinal epithelium, subsequent cell-to-cell spread, and manipulation of the host immune system (487). In addition to virulence plasmid acquisition, extensive pseudogenization of chromosomal genes has characterized the evolutionary progression of *E. coli* to *Shigella* (170; 576). Genes either unnecessary or inhibitory to the new intracellular lifestyle of *Shigella* were discarded (62).

The *Shigella* entry region (SER), an approximately 31 kb region of pINV, contains the *ipa/mxi/spa* operons, which encode the structural components of the T3SS needle and the translocon required for entry, in addition to several chaperone proteins (IpgA, IpgC, IpgE, and Spa15) necessary for proper folding of effector proteins during translocation through the T3SS (219; 341; 390; 400; 408; 410; 415; 477). The SER is both necessary and sufficient for invasion of *Shigella* into the host cell, and SER gene

expression is controlled through a regulatory cascade by the transcriptional activators VirF and VirB (283; 540; 572). *virF* is located outside the SER on pINV, while *virB* is encoded within the SER itself. A member of the AraC family of transcriptional regulators, VirF binds to and induces expression of *virB* and *icsA*, a pINV gene located outside the SER (540). Subsequently, VirB activates the *ipa*/*mxi*/*spa* promoters, as well as non-SER genes including *ospC2*, *ospC3*, *ospC4*, *ospD2*, *ospZ*, and *icsP* (42; 97; 313; 572).

Following host cell entry, roughly 20 additional effectors, encoded outside the SER, function in post-entry processes such as manipulation of the host immune system. Several members of the Osp and IpaH protein families, for example, modulate the host inflammatory response to promote successful bacterial colonization and dissemination (23; 31; 293; 319; 386; 452; 458; 471; 501; 523).

The ability of *Shigella* to spread from cell to cell is an essential post-entry process that allows for bacterial dissemination through the host epithelium. The outer membrane protein IcsA mediates motility of *Shigella* through the host cell cytoplasm and into neighboring cells (200; 201). To induce polymerization of host cell actin and stimulate bacterial motility, IcsA recruits and activates the host neuronal Wiskott-Aldrich syndrome protein (N-WASP), which in turn activates the Arp2/Arp3 complex to bind G-actin (145; 525). Although the *icsA* gene is located on the large virulence plasmid of *Shigella*, IcsA is not secreted through the T3SS, but functions as an autotransporter (76). Meanwhile, the T3SS effectors VirA, IcsP, and PhoN2 all contribute to *Shigella* cell-to-cell spread (20; 52; 144; 289; 493; 512). VirA activates host calpain proteases, which induce remodeling of the host cytoskeleton to form structures such as lamellipodial

protrusions, promoting bacterial spread (52). The bacterial protease IcsP restricts IcsA accumulation to the bacterial pole, consequently preserving directional motility (512). Finally, PhoN2 assists in the exposition of IcsA, possibly by binding to and inducing conformational changes of the outer membrane protein OmpA (493).

Although considerable work has been done to identify and describe the *Shigella* T3SS effectors responsible for post-entry events (for a review, see (487)), a handful of effectors remain largely uncharacterized. Traditionally, two primary methods have been employed to study the effect of bacterial proteins on eukaryotic cells. Transfection of bacterial genes into host cells provides an opportunity to study the phenotypic effects of a single bacterial effector upon the eukaryotic host cell. There are several limitations with this method, however: many host cell lines are particularly resistant to transfection, and the amount of bacterial protein produced within the cell is difficult to control, potentially leading to artificial or lethal effects due to improper expression (508). Furthermore, numerous T3SS effectors work together to exert their effects within the host cell, and solitary expression of these effectors may produce artificial or incomplete phenotypes (189). Alternatively, full gene deletion mutants can be generated for each effector gene and the corresponding phenotype loss demonstrated and later restored through genetic complementation. However, many T3SS effectors display functional redundancy (189). For example, all *Shigella* strains encode multiple copies of chromosomal and plasmid *ipaH* alleles that contribute to suppression of the inflammatory response during infection (31; 81). However, single-gene *ipaH* deletion mutants produce no virulence defects in a mouse model of infection; only when all *ipaH* genes are deleted (Δ*ipaH*-null mutant) does a phenotype emerge (31). IpaH9.8, IpaH4.5, and IpaH0722 have been characterized

as E3 ubiquitin ligases with substrate specificity for different host proteins of the NF-κB pathway (29-31; 570). Deletion of a single *ipaH* gene, therefore, is not sufficient to restore inflammation, as the remaining IpaH proteins act on other parts of the pathway to block NF-κB activation. When multiple genes contribute to the same phenotype, the individual virulence roles of each gene can become more difficult to ascertain. Functional redundancy appears to be a common trait of T3SS effectors, as *Salmonella*, *Pseudomonas*, and *Yersinia* all encode redundant T3SS effectors (9; 551; 609).

To bypass some of these challenges and develop a more suitable tool to study the contributions of various *Shigella* effectors to different virulence phenotypes, we employed a reductionist strategy. The SER was cloned into a fosmid backbone, along with the transcriptional activators of the *Shigella* T3SS (*virF*/*virB*). The resulting construct, termed the *Shigella* minimally invasive plasmid (*S*mip), encodes the minimal genes required to construct and regulate the T3SS needle structure and translocon complex needed for entry into host cells. T3SS effector genes can be added back to this system alone or in combination to examine their function in the context of bacterial infection. In this study, we characterize the *Shigella* virulence phenotypes of a pINV-cured strain carrying the *S*mip and demonstrate a proof of principle example for utilizing this tool by establishing the contributions of *icsA* and *virA* to *Shigella* intracellular spread.

METHODS AND MATERIALS

Strains

All strains and plasmids used in this study are listed in Tables 2 and 3, respectively. *S. flexneri* 2a strain 2457T and its derivatives were routinely grown in Trypticase soy broth (TSB) or on TSB plates with 1.5% agar and 0.25% Congo Red (Sigma) (TSB-CR).

BS103 is 2457T cured of the virulence plasmid, while BS766 is 2457T carrying the plasmid pKM208, which encodes genes for the lambda red recombination system (379). *E. coli* DH5α was used for cloning purposes, and *E. coli* EPI300 was used for propagation of pCC1FOS and its derivatives to high copy number using induction solution (Epicentre) in Luria-Bertani media. HeLa cells were grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in the presence of 5% CO_2. When required, ampicillin (100 μg/ml), kanamycin (50 μg/ml), spectinomycin (100 μg/ml) and/or chloramphenicol (12.5 μg/ml) were added to the growth media.

Construction of the *S*mip (Figure 5)

Cloning of* virF *into pCC1FOS.

Primers VirFFwd and VirFRev (Table 4) were used to amplify the wild type *virF* open reading frame (ORF) including the promoter and regulatory regions (an additional 406 bp upstream of *virF*) and to insert BlpI and Eco47III restriction sites at the 5' and 3' ends, respectively. The resulting PCR product was cloned into pGEMT (Promega), sequenced to confirm, and finally subcloned into the corresponding restriction site in pCC1FOS (Cm^R) (Epicentre), creating pYD56.

Cloning of the* Shigella *Entry Region (SER) (delineated by* ipaJ *and* orf131b*).

The method of Datsenko and Wanner (117) was performed in BS766 to insert a SfiI restriction site and a chloramphenicol resistance gene (SfiI-*cat*) into the 5' end of the SER (567 bp upstream of the *ipaJ* start codon) and another SfiI restriction site and a kanamycin resistance gene (SfiI-*kan*) into the 3' end (6 bp downstream of the *orf131b* stop site). Briefly, primers SERUP1 and SERUP2 (Table 4) were used to amplify *cat* using pKD3 as a template. SERUP1 contained a 5' overhang homologous to -523 to -484

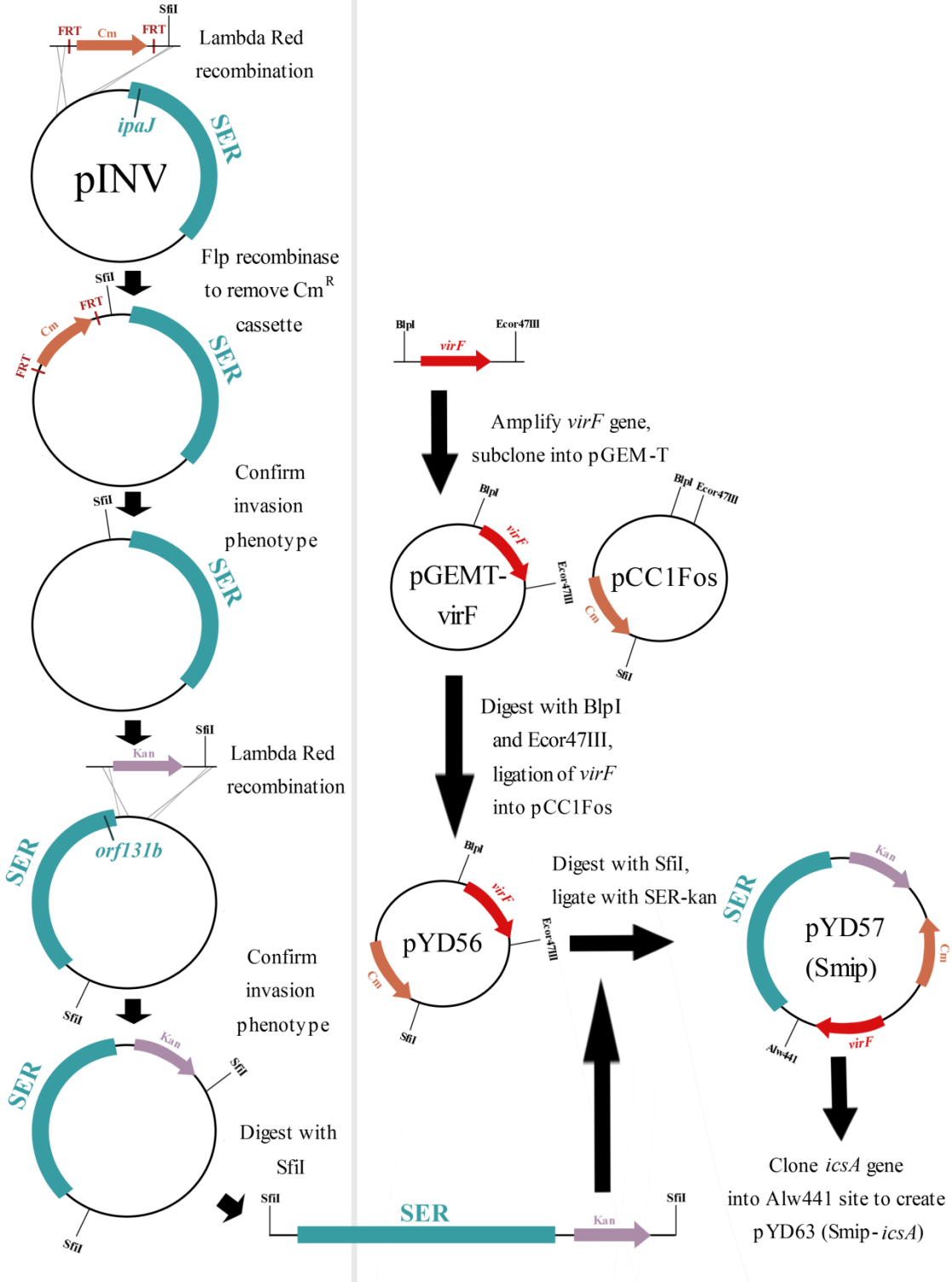
SfiI
FRT
Cm
FRT
Lambda Red recombination
ipaJ
SER
pINV
Flp recombinase to remove Cm^R cassette
Confirm invasion phenotype
Kan
Lambda Red recombination
orf131b
Confirm invasion phenotype
Digest with SfiI
BlpI
Ecor47III
virF
Amplify virF gene, subclone into pGEM-T
pGEMT-virF
pCC1Fos
Digest with BlpI and Ecor47III, ligation of virF into pCC1Fos
pYD56
Digest with SfiI, ligate with SER-kan
pYD57 (Smip)
Alw44I
Clone icsA gene into Alw441 site to create pYD63 (Smip-icsA)

Figure 5. Construction of the *S*mip.

For practical purposes, neither plasmids nor genes are drawn to scale, and only genes, enzymes, and sites pertinent to construction of the *S*mip are displayed. None of the genes in the *Shigella* entry region (SER) are specified except *ipaJ* and *orf131b*, which are noted during certain steps for orientation.

Table 2. Strains used in this study.

	Relevant genotype	**Source or reference**
Strains of *E. coli*		
DH5α	*F⁻ endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZΔM15 hsdR17*	Laboratory collection
EPI300	*F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 recA1 endA1 araD139 Δ(ara, leu)7697 galU galK λ⁻ rpsL (Str^R) nupG trfA dhfr*	Epicentre
Strains of *Shigella flexneri* 2a		
2457T	Wild type *S. flexneri* 2a	(181)
BS103	2457T cured of pINV	(341)
BS766	2457T/pKM208	(610)
BS856	Cm^R transductant of 2457T with P1 made on BS866	This study
BS858	BS856 with *cat* removed using FLP recombinase expressed from pCP20	This study
BS861	Kan^R transductant of BS858 with P1 made on BS867	This study
BS866	SfiI site and *cat* cassette introduced by λred into pINV of BS766 upstream of *ipaJ*, Cm^R	This study
BS867	SfiI site and *kan* cassette introduced by λred into pINV of BS766 downstream of ORF131b, Kan^R	This study
BS857	Kan^R transductant of 2457T with P1 made on BS867	This study
BS875	BS103/*S*mip (pYD57)	This study
BS889	BS103/*S*mip-*icsA* (pYD63)	This study
BS895	BS889/pYD68	This study

Table 3. Plasmids used in this study.

Plasmids		
pKD3	*ori*R6K *bla cat*	(117)
pKD4	*ori*R6K *bla kan*	(117)
pKM208	Ts *red-*, *gam-*, *lacI*-expressing plasmid under P_{tac} promoter; Amp^R	(379)
pCP20	FLP^+ λ*c*I857$^+$ λP_R +Rep^{ts} , Amp^R, Cm^R	(117)
pCC1FOS	Fosmid, *oriF* and *oriV* Cm^R	Epicentre
pGEMT	Cloning vector, Amp^R	Stratagene
pAM238	pACYC replicon, $Spec^R$	(58)
pYD56	pCC1FOS/ *virF*, Cm^R	This study
pYD57	SER in pYD56, Cm^R, Kan^R	This study
pYD59	P_{icsA}-*icsA'* in pGEMT, Amp^R	This study
pYD60	*'icsA* in pGEMT, Amp^R	This study
pRCS10	*icsA* cloned into EcoRI/SalI sites of pBAD18, Amp^R	Laboratory collection
pYD61	*'icsA* cloned into BlpI/SphI sites of pRCS10, Amp^R	This study
pYD62	P_{icsA}-*icsA'* cloned into EcoRI/XbaI of pYD61, Amp^R	This study
pYD63	P_{icsA} and entire *icsA* from pYD62 cloned into Alw44I site of pYD57, Cm^R, Kan^R	This study
pYD68	P_{virA}-*virA* cloned into HindIII/PstI sites of pAM238, $Spec^R$	This study

Table 4. Primers used in this study.

Primer Name	Sequence (5' to 3')[α]	Purpose	Forward or Reverse
VirFFwd	ATGCTGAGCGGGAGAATCGTCAATGCGGC	Amplify *virF* gene to clone into *S*mip	Forward
VirFRev	TCAGCGCTAAACCCATCTGGCAATAGCG	Amplify *virF* gene to clone into *S*mip	Reverse
SERUP1	GTACAACAGATTGAATGAACTGGACATGTTGATAGACGGCCGCCCGGGCCGTGTAGGCTGGAGCTGCTTC	Amplify *cat* cassette to clone into SER	Forward
SERUP2	CGCAGGTACCACCATGCTTACTTAATACTCAGAAGGCGAACATATGAATATCCTCCTTAGTTCC	Amplify *cat* cassette to clone into SER	Reverse
SERLP1	AAAGGAGAATAGCACGAAAGATACCAAATGATTAGCACTTGTGTAGGCTGGAGCTGCTTC	Amplify *kan* cassette to clone into SER	Forward
SERLP2	AAATTACAGTCTCAAAACAAACCCAACGGTCAATATAGTTGGCCCGGGCGGCCCCATATGAATATCCTCCTTAGTTCC	Amplify *kan* cassette to clone into SER	Reverse
pYA90F	GAATTCGTGCACTCGAGGCATTGGAGAGTCTGGTAGGAAAG	Amplify head of *icsA* gene to clone into *S*mip	Forward
pYA90R	GTCACCACCATTACCGCCACAAC	Amplify head of *icsA* gene to clone into *S*mip	Reverse
pYA91F	ATGACCGACACGGTAGCACTATTG	Amplify tail of *icsA* gene to clone into *S*mip	Forward
pYA91R	GCATGCGTGCACGGCGCGCCACGGTGGGTCCCAGAGAAATG	Amplify tail of *icsA* gene to clone into *S*mip	Reverse
pYA94F	CAAGCTTCTTTCCTACCAGACTCTCCAATGC	Amplify *virA* gene to clone into pAM238	Forward
pYA94R	CCTGCAGGGATGGGCTGAGATTCTATTGGTC	Amplify *virA* gene to clone into pAM238	Reverse
[α]Restriction sites underlined.			

from the start codon of *ipaJ*, with a SfiI restriction site included, while SERUP2 primer contained sequence homologous to -433 to -394 from the start codon of *ipaJ*. The PCR fragment amplified by the two primers was introduced into BS766 and Cm^R colonies were isolated to select for recombinants. This procedure resulted in the insertion of a SfiI restriction site and the cat^R marker replacing the 50 basepair region between -484 and -433 upstream of *ipaJ* start codon. The insertion was then transduced into a clean background of 2457T (creating BS856), followed by the removal of the *cat* cassette using the plasmid pCP20 to create BS858. Invasion assays were performed to ensure that the insertion did not affect the expression of genes encoding T3SS effectors in the SER. Using the SERLP1/SERLP2 primers (Table 4) and pKD4 as a template, the same method was used to introduce another SfiI restriction site and a kan^R marker to the 3'end of SER (6 bp downstream of the *orf131b* stop site), creating BS867. P1 lysate grown on BS867 was used to transduce the Sfi site and the kan^R marker into BS858, creating BS861, in which the 31 kilobase SER is flanked by SfiI sites and a kan^R marker inserted at the 3'end. Invasion assays were performed to again ensure that this insertion did not negatively affect T3SS activity.

***Isolation of SER-Sfi-kan and its cloning into pCC1FOS*-virF**

A large scale preparation of virulence plasmid DNA from BS861 was performed using a modified procedure of Kado and Liu (282) followed by a $CsCl_2$ gradient as previously described (225). The virulence plasmids were digested with SfiI, run on 0.8% GTG agarose at 35V overnight, and a slice of agarose corresponding to 30-40 kb in size was isolated. The agarose was removed by treatment with Gelase (Epicentre) following the manufacturer's protocol and the DNA was isolated by ethanol precipitation. The digested

virulence plasmid DNA was subsequently ligated to the SfiI-digested pYD56 (pCC1FOS-*virF*). The ligation mixture was packaged into MaxPlax lambda particles (Epicentre). After infection of *E. coli* EPI300 and selection on LB_{KanCm}, the resulting strain, ATM949, was isolated. PCR was performed on this strain to confirm the presence of genes in the SER. The resulting plasmid, pYD57, now called *S*mip, was isolated from ATM949 after induction to high copy, using the Qiagen Large Construct Kit (Qiagen). Electroporation of pYD57 into BS103, and selection on LB_{KanCm}, yielded BS875.

Cloning of icsA *into the* S*mip*

The *icsA* ORF and its upstream regulatory region (3771 bp in total) were obtained by ligation from three fragments: the head (-370 to the XbaI site in the *icsA* ORF), which was amplified with pYA90F/pYA90R primers (Table 4); the central part of the *icsA* ORF (from the XbaI to BlpI sites), which was obtained from pRCS10; and the tail (from the BlpI site in *icsA* ORF to the *icsA* stop codon), which was amplified with pYA91F/pYA91R primers (Table 4). The head and the tail portions were cloned separately into pGEMT (Stratagene), sequenced, and then subcloned into pRCS10 using the EcoRI-XbaI and BlpI-SphI sites, respectively. Once the head, central and tail parts were assembled, the full-length *icsA* was cloned into the *S*mip at the Alw44I site. In order to accommodate the sequential subclonings, EcoRI, Alw44I, and XhoI were added in the forward primer pYA90F (Table 4), while AscI, Alw44I, and SphI sites were added to pYA91R primer (Table 4). EcoRI and SphI were used for cloning the respective head and the tail portions into pRCS10; Alw44I was used to subclone the entire 3771 bp fragment into the *S*mip; and XhoI and AscI were included to insert additional single restriction

sites into the *S*mip for future cloning purposes. The resulting plasmid (pYD63) was called *S*mip-*icsA*, and was subsequently transformed into BS103 to yield BS889.

Cloning of virA

The *virA* ORF and its promoter region (1474 basepairs total) were cloned into the HindIII/PstI sites of pAM238 using the pYA94F/pYA94R primers (Table 4). The plasmid was introduced into BS889 by electroporation.

Invasion and intracellular growth assays

Bacteria were used to infect nonconfluent monolayers of HeLa cells as previously described, with some modifications (224). Briefly, both HeLa cells and bacteria were washed with 1x PBS, then bacteria were resuspended in 1x DMEM and approximately 2 x 10^8 mid-log phase bacteria were added to each 35 mm well of HeLa cells. Plates were centrifuged at 3,000 rpm for 10 minutes to facilitate cell invasion, and then incubated at 37°C for 30 minutes. Cells were washed with 1x PBS, treated with 50 µg/mL gentamicin in 1x DMEM to kill any extracellular bacteria not removed by the wash, then incubated for an additional 30 minutes at 37°C. Cells were washed again, treated with DMEM/gentamicin, and incubated for a final 60 minutes at 37°C. HeLa cells were lysed with 0.5% Triton X-100 and viable bacteria were titered by plating on TSB-CR and colony counts taken following overnight incubation at 37°C. The percent efficiency of invasion was calculated for each strain ((bacterial output/bacterial input) x 100). To determine intracellular growth, a similar method was utilized, except HeLa cells were lysed at 0.5, 1.5, 2.5, and 3.5 hours following initial infection.

Congo Red secretion assay

Secretion of T3SS effectors was measured as previously described (610). Briefly, 25 mL of each bacterial strain was grown to late log phase at 37°C, normalized to the same OD_{600} to ensure equal amount of bacteria, and incubated in 1x PBS with 30 µg/mL Congo Red (CR) for 1 hour at 37°C to facilitate secretion. Bacteria were centrifuged and bacterial pellets (whole cell lysates) and supernatants (secreted proteins) separated. Whole cell lysates were resuspended in 500 µL 1x Laemmli buffer. Supernatant fractions were filtered with a 0.2 micron filter (Millipore) to eliminate any remaining bacteria, and then precipitated with 10% trichloracetic acid (TCA) for 1 hour followed by an acetone wash. Pelleted protein was resuspended in 50 µL 1x Laemmli buffer. Both whole cell lysate and supernatant fractions were boiled for 5 minutes, and then run on 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels. Proteins were then transferred to nitrocellulose membranes, and blotted overnight with 1:1000 dilution of mouse polyclonal anti-IpaC antibody (a gift from Edwin Oaks, Walter Reed Army Institute of Research). Blots were washed, and then incubated with a secondary anti-mouse antibody conjugated to horseradish peroxidase (Amersham Biosciences, 1:5000 dilution). Blots were developed using the ImageQuant^{TM} LAS 4000 according to manufacturer's instructions. Densitometry analysis was performed using the ImageQuant TL software, and the percent secretion for IpaC calculated ((secreted IpaC protein/total IpaC protein) x 100).

Immunofluorescence assays

To visualize the recruitment of cellular actin, HeLa cells were seeded on coverslips and infected with approximately 1 x 10^9 bacteria grown to mid-log phase. The plates were centrifuged at 3,000 rpm for 10 minutes to facilitate cell invasion and incubated at 37°C

for either 30 minutes to visualize actin remodeling following invasion, or for 2 hours to observe bacteria associated with actin tails. The cells were then fixed (1x PBS, 3% paraformaldehyde and 3% sucrose) for 10 minutes at room temperature, permeabilized with 0.2% Triton X-100 for 5 minutes, and blocked in 1X DMEM/5% FBS for 30 minutes. Bacteria were visualized with a rabbit anti-*Shigella* antibody (Life Span LS-C124528; diluted 1:100) and then with an Alexa 488-labelled goat anti-rabbit antibody (Molecular Probes; 1:1000). Actin was stained with rhodamine–phalloidin for 30 minutes (Sigma; diluted 1:500). Finally, coverslips were mounted in fluorescence-mounting medium and analyzed with an Olympus IX81 microscope. For IcsA staining, bacteria were grown to mid-log phase and fixed to poly-lysine-treated coverslips as described above. The coverslips were then blocked in 1x PBS/5% FBS for 30 minutes and IcsA was stained using a mouse monoclonal antibody against IcsA (473) (diluted 1:200) overnight at 4°C in 1x PBS/5% FBS and a secondary goat anti-mouse antibody conjugated to Alexa 488 (Molecular Probes; diluted 1:1000).

Plaque assay

Plaque assays were performed as previously described (396). HeLa cell monolayers were grown for 3-4 days in 60 mm dishes to obtain a confluent monolayer. Overnight cultures of bacteria were diluted 1:50 in TSB and grown for 2 hours. Cells were infected with either 1×10^4 or 1×10^3 bacteria for 2 hours at 37°C with 5% CO_2. An agarose overlay containing 1x DMEM and 20 µg/ml gentamicin was applied to infected monolayers, and the plates were incubated at 37°C in a 5% CO_2 atmosphere for five days. Neutral red staining allowed for determination of the plaque diameters. Plaque diameters were taken from 10 plaques per experiment over the course of three independent experiments.

RESULTS

Construction of the *S*mip

To mimic copy number of the *Shigella* large virulence plasmid and prevent potentially artificial events from a mid- or high-copy number plasmid, a single copy fosmid (pCC1Fos) was used as the backbone for the *S*mip (309). First, the *virF* T3SS transcriptional regulator gene was cloned into pCC1Fos. Next, lambda red-mediated linear recombination methods (117) were used to insert an antibiotic resistance marker at one end of the 31 kb *Shigella* entry region (SER), and to specifically tag both ends of SER with SfiI restriction sites, which facilitated the cloning of the entire SER region into pCC1Fos-*virF*. The resulting plasmid, the *S*mip, contains all the genes required for invasion of the eukaryotic host cell, in addition to the transcriptional activators *virF* and *virB*. The *S*mip carries roughly 35 kb of the *Shigella* virulence plasmid, and therefore constitutes a contracted version of the 220 kb large virulence plasmid of *Shigella*. The *S*mip was electroporated into BS103, a derivative of *S. flexneri* 2a strain 2457T cured of the large virulence plasmid, to create BS875.

To develop a *S*mip strain capable of minimal cell-to-cell spread, a second version of the *S*mip, termed *S*mip-*icsA*, was also constructed (Figure 6). Two additional restriction sites, XhoI and AscI, were also added into *S*mip-*icsA* to assist future cloning projects. *S*mip-*icsA* was electroporated into BS103 to create BS889.

Bacterial invasion and intracellular replication in HeLa cells

Functionality of the T3SS is dependent upon expression of the T3SS genes, proper assembly of the needle structure, and secretion of several effectors, including the invasins IpaB and IpaC (563). To verify that the *S*mip minimal T3SS was functional, the

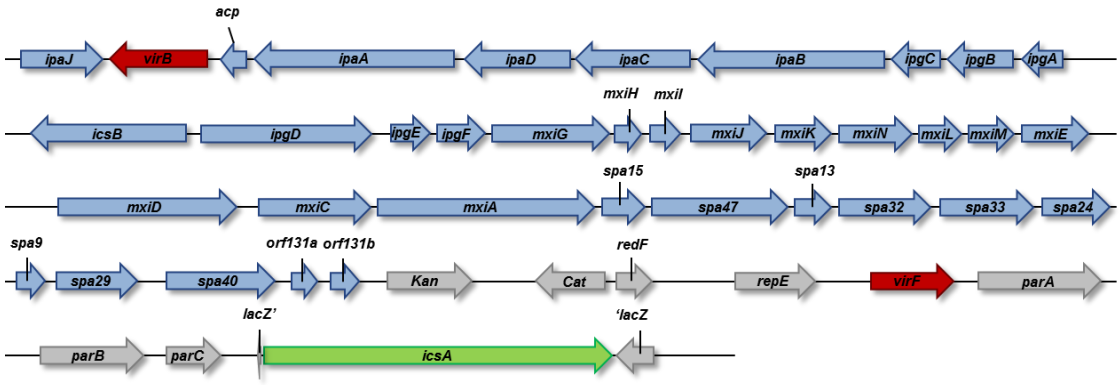

Figure 6. *S*mip-*icsA* plasmid map (linearized).

The *S*mip contains the *Shigella* entry region (SER, blue), which encompasses the structural T3SS genes, the transcriptional activators *virF*/*virB* (red), and the pCC1Fos backbone genes (gray) required for plasmid maintenance and replication. Two origins of replication, the multi-copy *oriV* and single-copy *oriF*, are also present on the backbone (not shown). As initiation of replication from *oriV* requires addition of the *trfA* gene product which is absent in *Shigella*, the *S*mip is present at single-copy number in *Shigella*. *icsA (green)*, the autotransporter required for actin polymerization and cell-to-cell spread, was additionally cloned onto the *S*mip to create *S*mip-*icsA*.

ability of BS875 to invade host cells was examined. A gentamicin protection assay was performed on HeLa cells infected with strains 2457T, BS103, and BS875 (Figure 7). As expected, BS103 was noninvasive, confirming the necessity of a functional T3SS in *Shigella* invasion. BS875, meanwhile, was able to invade at approximately 60% of the wild type 2457T strain, demonstrating that the T3SS apparatus was present and functional on the surface of these bacteria. Therefore, the minimal T3SS encoded on the *S*mip is sufficient to restore invasion in a plasmid-cured strain of *Shigella*, although the lower invasion efficiency indicates that perhaps other virulence factors encoded outside the SER are required for the full invasive phenotype.

Following entry, *Shigella* lyse the endosome and escape to the cytoplasm of the infected cell to replicate, a mechanism dependent on the concerted actions of IpaB, IpaC, and the chaperone IpgC (409). All three of the genes encoding these proteins are encoded on the *S*mip; therefore, BS875 should be able to escape the endosome and replicate within the host cell. To verify this hypothesis, the intracellular replication of 2457T and BS875 was accessed (Figure 8). Both strains multiplied equally well in HeLa cells over the first four hours post-infection, with no significant differences between strains. Since *Shigella* escape from the endosome is a prerequisite for bacterial replication in the cytoplasm, these results indicate that BS875 is capable of endosome lysis (407). Taken together, these data demonstrate that BS875 is capable of invasion, endosome lysis, and replication within the host cell.

Secretion of T3SS effectors

Although the invasive phenotype of BS875 indicated that the *S*mip minimal T3SS was assembled and functional, the invasion efficiency was slightly lower compared to the

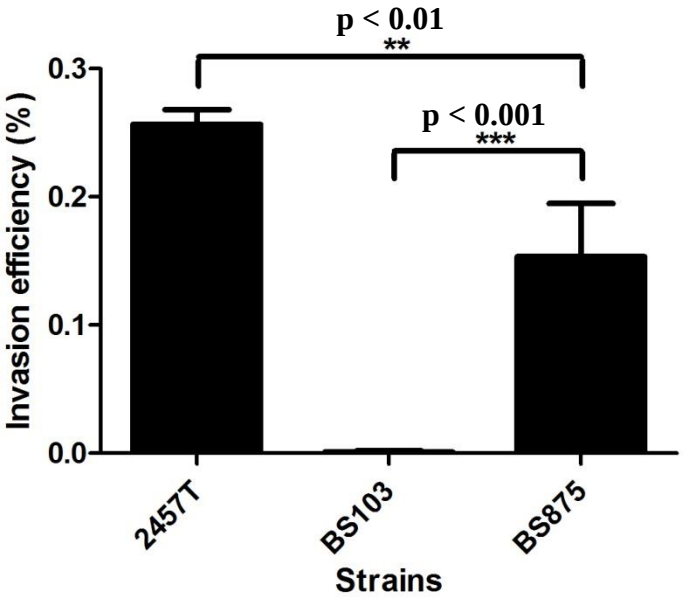

Figure 7. Invasion of *S. flexneri* 2457T, BS103, and BS875.
A gentamicin protection assay was performed on HeLa cells infected with *S. flexneri* 2457T, BS103, or BS875. The mean percent efficiency of invasion was calculated for each strain ((bacterial output/bacterial input) x 100). Error bars indicate ± one standard deviation from the mean over three independent experiments.

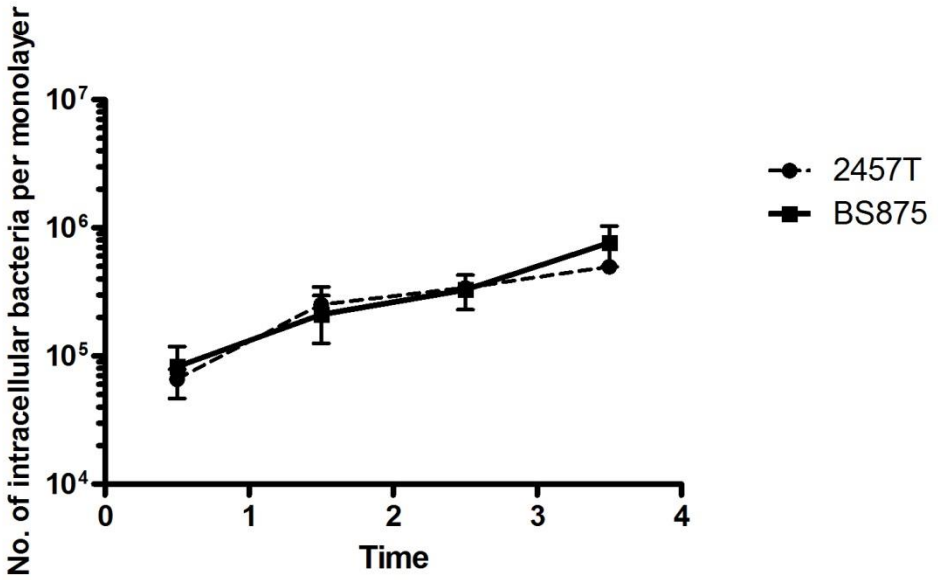

Figure 8. Intracellular replication of *S. flexneri* 2457T and BS875.
HeLa cells were infected with wild type 2457T or BS875 and lysed with Triton X-100 at 0.5, 1.5, 2.5, or 3.5 hours post-invasion. The data shown are one representative experiment performed in triplicate. Error bars indicate ± one standard deviation from the mean.

wild type strain. To confirm that this defect was not due to decreased secretion of the T3SS invasins, a Congo Red secretion assay was performed. Congo Red, a synthetic sulfonated azo dye, is an artificial inducer of *Shigella* type three secretion *in vitro*, and is thought to mimic *in vivo* T3SS contact with the host cell (36). After one hour induction with Congo Red, approximately 70% of the total T3SS effector IpaC was present in the supernatants of 2457T and BS875 (Figure 9). Total IpaC protein was also equivalent (data not shown). The lack of any significant difference in total or secreted IpaC between these strains indicates that the reduced BS875 invasion phenotype is not due to an impaired ability of this strain to produce or secrete the T3SS invasins.

Intercellular spread

Shigella cell-to-cell spread is essential for bacterial dissemination through the host epithelium. A common *in vitro* measure of intercellular motility is the ability of a strain to form plaques on an epithelial cell monolayer (53; 396). The *Shigella* virulence plasmid genes that contribute to intercellular spread include *icsA*, *icsB*, *icsP*, *virA*, and *phoN2* (20; 52; 53; 144; 289; 493). The *S*mip construct lacks all of these genes, as they are encoded outside of the SER on the large virulence plasmid. Unsurprisingly, BS875 is deficient in cell-to-cell spread, as indicated by the inability of this strain to generate visible plaques on HeLa cell monolayers (Figure 10). Furthermore, when host F-actin filaments of infected cells were stained with rhodamin-phalloidin, it is apparent that BS875 was unable to polymerize host actin at the bacterial pole or form distinct ‘comet tail’ protrusions characteristic of the wild type strain, 2457T (Figure 11).

As a proof of principle demonstration of the *S*mip as a useful genetic tool, the contributions of the virulence plasmid genes *icsA* and *virA* to *Shigella* intercellular spread

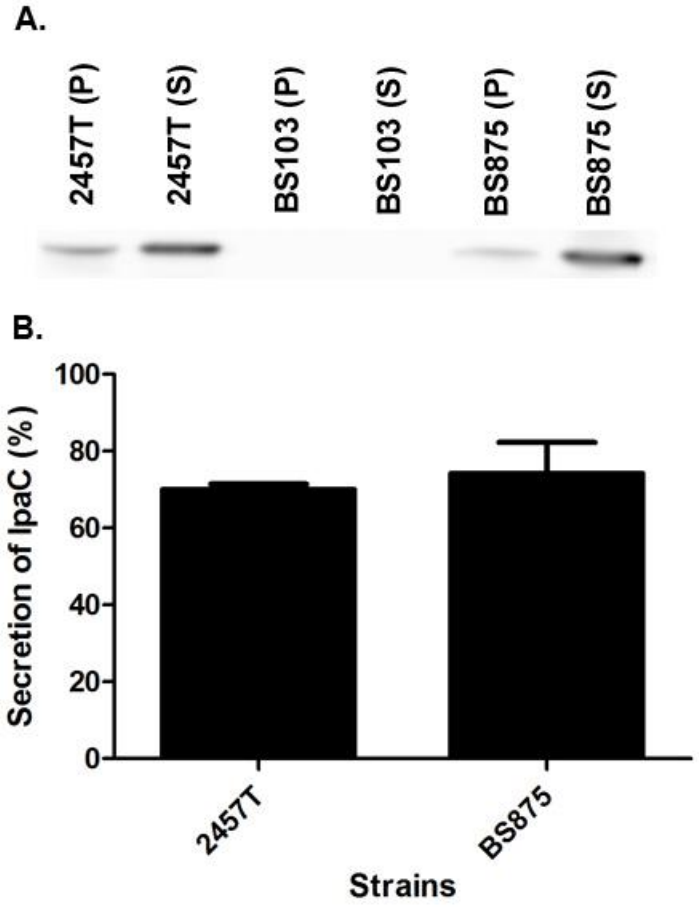

Figure 9. T3SS effector secretion of *S. flexneri* 2457T, BS103, and BS875. Congo Red secretion assays were performed, and the pellets and supernatants were analyzed on an SDS-PAGE gel. (A) Representative Western blot analysis of IpaC. Expression of IpaC was only detected in strains carrying a T3SS (2457T and BS875). (B) Following densitometry analysis, the percent secretion of IpaC was calculated for 2457T and BS875 ((secreted IpaC/total IpaC) x 100). Each value represents the mean ± one standard deviation over three independent experiments. There was no significant difference in secretion between these strains (unpaired t-test).

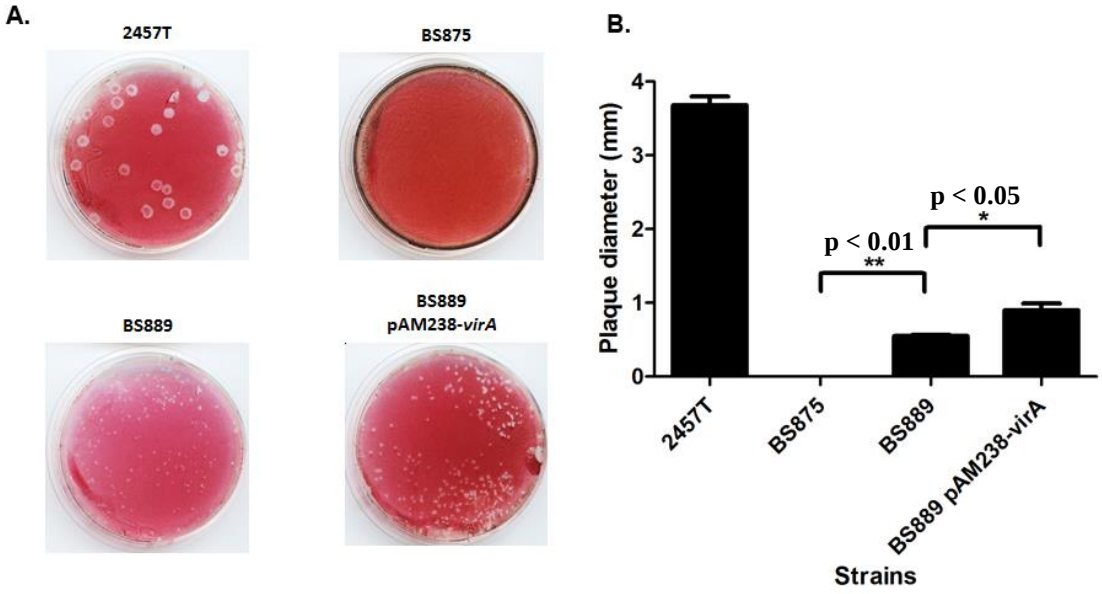

Figure 10. Ability of the *Shigella*/*S*mip strains to spread from cell to cell. Representative plaques formed by *S. flexneri* 2457T, BS875, BS889, and BS889 pAM238-VirA. (B) Measure of the plaque diameters (in mm) generated for each strain. Error bars represent the mean ± one standard deviation over three independent experiments.

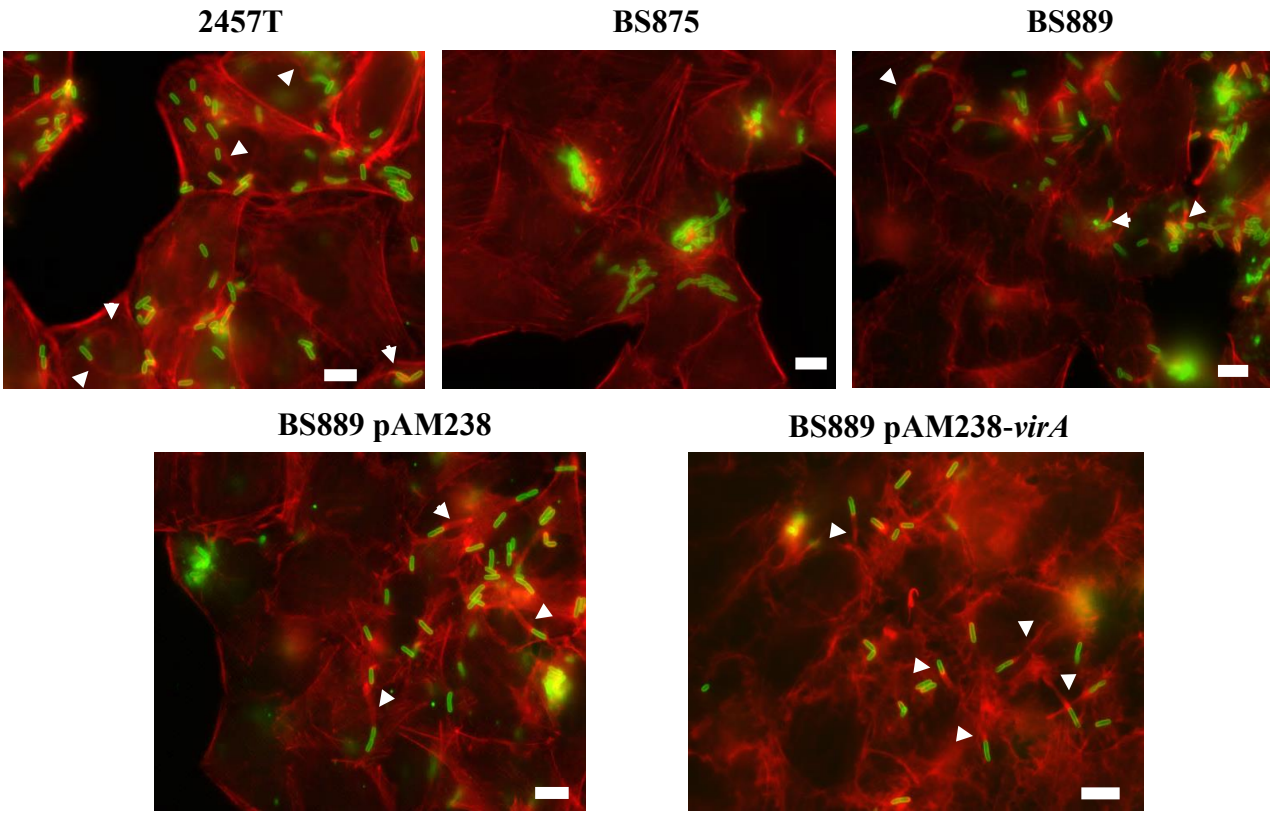

Figure 11. Visualization of actin tail formation during cell-to-cell spread of *S. flexneri*. HeLa cells were infected for two hours at 37°C and then processed for immunofluorescence. Actin was stained with rhodamin-phalloidin (red) and bacteria with anti-*Shigella* antibody (green). The scale represents 10 μm. Arrows indicate bacteria associated with an actin tail.

were demonstrated. The ability of *Shigella* to polymerize host actin is an essential component of intercellular motility, as a transposon insertion into the *icsA* gene renders the bacteria completely attenuated for cell-to-cell spread (53). To explore only the contribution of IcsA to intercellular spread in the *S*mip background, the *icsA* gene was cloned into the *S*mip plasmid to create *S*mip-*icsA*, and subsequently transformed into BS103. The resulting strain, BS889, formed pinpoint plaques in the plaque assay (Figure 10). The plaque size created by BS889 strain was roughly 15% of the wild type, indicating that although IcsA is necessary for cell-to-cell spread, it is not sufficient for the full phenotype. BS875, which does not carry *icsA*, was unable to recruit actin and bacteria were grouped in microcolonies within the cell. In contrast to BS875, BS889 was surrounded by actin filaments and distinct actin 'comet tails' were present behind several bacteria in infected host cells. However, comet tail formation was rare compared to those produced by wild type *S. flexneri* (Figure 11).

Finally, a strain expressing both *icsA* and *virA* (BS889 pAM238-*virA*) was constructed. VirA, a member of the EspG protein family from enteropathogenic *E. coli*, promotes calpastatin degradation, indirectly activating host calpain proteases (52). Calpain activation prompts the translocation of these proteins to the plasma membrane, where they participate in host cytoskeleton remodeling (429). During *Shigella* infection, VirA is hypothesized to induce lamellipodia formation and facilitate cell-to-cell spread, as strains with random transposon insertions into the *virA* gene are attenuated in plaque formation (52; 553). Plaque size of the strain expressing both *icsA* and *virA* in the *S*mip background was approximately 24% of wild type, a significant increase over the strain expressing *icsA* alone (Figure 10). These data confirm the individual contributions of

icsA and *virA* to intercellular spread, and support the *S*mip as a functional tool for continued exploration of *Shigella* T3SS effectors.

DISCUSSION

In this study, the *Shigella* minimally invasive plasmid (*S*mip), a novel tool for exploring the contribution of *Shigella* T3SS effectors to virulence, was constructed and characterized. The *Shigella* entry region (SER), which encompasses the *mxi*/*ipa*/*spa* genes necessary for T3SS invasion into host epithelial cells, was cloned into a single-copy fosmid backbone along with the primary T3SS transcriptional activator, *virF*. In total, 37 ORFs reside in the SER, including two additional T3SS transcriptional regulators, *virB* and *mxiE* (Figure 6). To generate a construct capable of inducing bacterial cell-to-cell spread, an alternative version of the *S*mip, *S*mip-*icsA*, was created by cloning the *icsA* gene into the *S*mip. BS103, a plasmid-cured strain of wild type *S. flexneri* strain 2457T, was transformed with the *S*mip and *S*mip-*icsA* constructs to create strains BS875 and BS889, respectively. The virulence phenotypes of these strains were characterized and the contributions of *icsA* and *virA* to intercellular spread were determined.

BS875 was capable of T3SS secretion, invasion, and intracellular replication, demonstrating that the T3SS encoded on the *S*mip was assembled and functional at the surface of the bacterium. Although secretion and intercellular replication were unchanged from the wild type 2457T strain, invasion was slightly attenuated for BS875, indicating that other genes on the major virulence plasmid but outside the SER contribute to optimal invasion.

The inability of BS875 to form plaques was also unsurprising, as none of the genes in the SER have been shown to mediate intercellular spread. BS889, which carries Smip-icsA, generated pinpoint plaques in the cell-to-cell spread assay, and furthermore was able to polymerize host cell actin and form comet tails, confirming previous work supporting the hypothesis that *icsA* is both necessary and sufficient for intercellular motility (53; 201). When *icsA* and *virA* were expressed in combination with the *S*mip genes, the resulting *Shigella* strain was significantly more capable of spreading from cell-to-cell over the *S*mip or *S*mip-*icsA* alone, likewise confirming the role of *virA* in cell-to-cell spread (52). However, expression of these two genes together did not completely restore plaque size to that of the wild type (2457T); additional T3SS proteins outside of the SER are required to optimize the cell-to-cell spread phenotype, including IcsP and PhoN2 (20; 144; 289; 493; 512). We hypothesize that combined expression of these effectors in the BS889 background will continue to increase intercellular spread (and hence, plaque size) until all genes required for the complete spreading phenotype are identified. Using the *S*mip to define genes responsible for intercellular spread is merely one possible application; other T3SS effectors can be investigated in this manner. In our laboratory, the *S*mip has already been utilized to characterize the T3SS effector OspC1 (Joseph *et al.*, manuscript in preparation).

Additionally, the *S*mip may be utilized to identify novel antivirulence genes (AVGs) in *Shigella*. An AVG is a gene that has been inactivated or deleted from all strains of a pathogen because expression of the AVG interferes with optimal virulence (62; 339; 342). Identification of these genes is crucial to understand the evolutionary history of niche-adapted pathogens, and may help to either uncover new virulence factors

or further define existing ones. AVGs have been most extensively studied in *Shigella* due to the extreme pseudogenization of this pathogen following their divergence from commensal *Escherichia coli* isolates, which provides clues to the history of previously-functional genes or gene systems. As a result, several *Shigella* AVGs, including *cadA*, *nadA*/*nadB*, *speG*, and *ompT* have been identified (39; 171; 342; 346; 381; 434). For the most part, the approach to classify new AVGs requires the expression of candidate AVGs in wild type *Shigella*; unfortunately, the identification of promising candidate AVGs is still largely hit-or-miss, as it is impossible to differentiate between a gene lost due to neutral selection (not an AVG) and a gene lost due to negative selection (an AVG). Our laboratory is currently exploring a potential alternative approach to AVG identification by transforming the *S*mip into commensal *E. coli* strains and studying the virulence phenotypes of the resulting strains. All strains tested have been very poorly invasive, indicating the presence of one or more AVGs in these isolates (data not shown). Targeted or random gene deletions in these strains, and subsequent testing for virulence restoration, could assist in the identification of novel AVGs.

The *S*mip could also aid in the development of a safe and effective *Shigella* vaccine, especially as BS875 successfully invades host cells, yet is attenuated for intercellular spread and lacks most of the post-invasion T3SS effectors necessary for full pathogenesis. Several live attenuated *Shigella* vaccine strains were previously tested in clinical trials. These strains harbor deletions in genes such as *icsA*, *guaBA* (quinine-nucleotide biosynthesis), *iuc* (aerobactin), *aroA*/*aroD* (aromatic amino acid biosynthesis), and/or the enterotoxin genes *sen* and *set* (287; 288; 300-302). When administered in healthy adult humans, however, many of these vaccines induced mild to severe adverse

reactions in a small population of vaccinees, including full manifestation of dysentery in certain individuals. Thus far, the least reactogenic vaccines have also been the most attenuated, including the *ΔicsA*/Δ*sen*/Δ*set*/Δ*guaBA* vaccine candidate (301). BS875 may prove to be an effective alternative to these live attenuated strains, potentially by decreasing the likelihood of adverse effects yet still providing immunogenicity. While the mechanisms of protection against *Shigella* infection are not yet fully understood, antibody development against the O- and Ipa-antigens is thought to be important for the generation of a protective adaptive immune response (115; 318; 397). Based on our results, BS875 produces and secretes the Ipa invasins (Figure 9). O antigen diversity forms the basis for serotype differentiation among *Shigella* strains, and studies indicate that cross-protection between infections with heterologous *Shigella* serotypes is limited (161; 318). In a 1991 study of children in Santiago, Chile, infection with one serotype was shown to confer high protection (72%) against subsequent infection with homologous serotypes, but insignificant protection against subsequent infection with a heterologous serotype (173). To complicate vaccine development efforts, serotype distribution varies worldwide. Although the majority of infections in the developing world are caused by the endemic *S. flexneri* serotypes 2a and 6, epidemics of the more lethal *S. dysenteriae* type 1 can also occur (303). In developed nations, *S. sonnei* strains are responsible for the majority of infections. Transformation of the *S*mip into plasmid-cured derivatives of the most medically relevant serotype backgrounds (*S. flexneri* 2a and 6, *S. sonnei*, and *S. dysenteriae* type 1) might therefore serve as a broadly protective live attenuated vaccine.

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CHAPTER 6: THE SEARCH FOR NOVEL AVGS IN *SHIGELLA*

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The data presented in this chapter are preliminary and will be submitted for publication upon completion of analysis.

K. A. Bliven is responsible for the work presented in this chapter, with the following exceptions: A.T. Maurelli assisted with experimental design and analysis.

Note: The figure, table, and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

The *Shigella* genus evolved from multiple lineages of *Escherichia coli*; in the process, harmless extracellular commensals developed into intracellular human pathogens capable of eliciting bloody diarrhea. The critical turning point in this evolutionary progression was the acquisition of a large 220 kb virulence plasmid (pINV), which harbors genes encoding a type three secretion system (T3SS) essential for host cell invasion and subsequent cell-to-cell spread. Conversely, *Shigella* also lost or inactivated genes that are inhibitory to the bacterium's new intracellular lifestyle. These genes are known as antivirulence genes (AVGs). To identify novel AVGs which target the *Shigella* T3SS, *S. flexneri* 2a genes encoding the T3SS, in addition to the T3SS transcriptional activators *virF* and *virB*, were expressed from their native promoters on a plasmid known as the *S*higella minimally invasive plasmid (*S*mip). The gene encoding IcsA, an autotransporter protein essential for *Shigella* cell-to-cell spread, was also cloned into the *S*mip. *S*mip-*icsA* (simply termed '*S*mip' for the purposes of this chapter) was introduced into four different commensal *E. coli* strains and a wild type *S. flexneri* 2a derivative (BS103) cured of the virulence plasmid. All *E. coli*/*S*mip strains were significantly attenuated for host cell invasion compared to BS103/*S*mip. RNA transcript data for the two T3SS regulators, *virF* and *virB*, as well as the T3SS effector *ipaC*, were collected. In *E. coli* SE11/*S*mip, transcript levels of *virB*, and the downstream *ipaC* gene, were significantly inhibited compared to BS103/*S*mip (17- and 23-fold reduction, respectively). This inhibition correlated with a decrease of total IpaC protein in a Congo Red secretion assay. Taken together, our results suggest that there is a novel inhibitor(s) of *virB* expression in *E. coli* SE11, and additional inhibitor(s) of invasion in the other *E. coli* backgrounds.

INTRODUCTION

A facultative intracellular bacterial pathogen, *Shigella* evolved directly from extracellular commensal *Escherichia coli* following acquisition of a large 220 kb virulence plasmid (pINV), which harbors genes encoding the T3SS necessary for host cell invasion (438). The results of two separate studies utilizing distinct techniques led Pupo *et al.* to hypothesize that this acquisition event took place not once, but at least four times during a period of 35,000 to 270,000 years ago (437; 438). In the first study, 32 pathogenic *E. coli* and *Shigella* strains were compared to the *E. coli* Reference Collection (ECOR), a set of primarily commensal *E. coli* strains originally chosen to represent genetic diversity in the *E. coli* genus (399; 437). Multi-locus enzyme electrophoresis (MLEE) and sequence analysis of the *mdh* housekeeping gene were performed on these isolates. Intriguingly, the *Shigella* strains cluster in not one, but several distinct groups within the *E. coli* genus. In a following study, sequence analysis of four highly conserved chromosomal regions in 46 strains representative of the *Shigella* genus confirmed that the *Shigella* isolates cluster together in at least four separate groups (438). Cluster 1 contains most of the *S. boydii* and *S. dysenteriae* strains, in addition to *S. flexneri* serovars 6 and 6A; cluster 2 consists of the remaining *S. boydii* strains and *S. dysenteriae* 2; and cluster 3 consists of the remaining *S. flexneri* strains. Although Pupo *et al.* did not assign *S. sonnei* to a separate cluster, they acknowledged that the *S. sonnei* strains did not group with any of the three main clusters, indicating that *S. sonnei* forms its own, independent group. Enteroinvasive *E. coli* (EIEC), which carry the same pINV present in *Shigella*, are less infectious, have a lower efficiency of intercellular spread, and produce a smaller inflammatory response during infection than *Shigella* isolates (139; 368). Phylogenetic analyses of EIEC, *Shigella*, and ECOR strains indicate that EIEC isolates also cluster in

at least four distinct groups within commensal *E. coli*; none of these groups were common to the *Shigella* clusters (308; 420). Peng *et al.* (2009) postulate that the emergence of the EIEC isolates occurred more recently than *Shigella*; phenotypically, EIEC strains share more biochemical properties with *E. coli*, such as the ability to utilize sodium acetate and ferment mucate (420; 498). If the EIEC strains have had less time to adapt to the intracellular niche, they may represent an intermediate phenotype between harmless commensal *E. coli* and the more pathogenic *Shigella*. Taken together, these data support the hypothesis that both *Shigella* and EIEC evolved from multiple independent lineages of commensal *E. coli*. Following acquisition of pINV, these strains were likely subject to similar selective pressures within a shared host environment, leading to convergent evolution in the *Shigella*/EIEC pathovar.

In addition to pINV acquisition, horizontal gene transfer of several chromosomal pathogenicity islands (SHI-1, SHI-2, SHI-3, SRL, and SHI-O) has also occurred in certain *Shigella* strains. SHI-1 encodes the ShET1 enterotoxin, the SigA cytopathic protease, and the Pic serine mucinase (16; 165; 239). The SHI-2 locus encodes an aerobactin iron transport system, the ShiD colicin immunity protein, and ShiA, an immunomodulator (371; 566). SHI-3, present only in strains of *S. boydii*, carries an aerobactin system highly similar to the one found in SHI-2 (440). The SRL locus carries a functional ferric di-citrate uptake system and a number of antibiotic resistance cassettes (328). Lastly, the SHI-O loci include a number of temperate bacteriophages (SfX, SfiI, and SfV) that modify the O polysaccharide side chain of the cell envelope lipopolysaccharide (LPS) (214; 259; 344). In contrast to pINV, however, none of these

PAIs are present in every strain of *Shigella*, and therefore do not contribute to the core *Shigella* genes essential for virulence.

Apart from the genes present on pINV and the chromosomal PAIs, *Shigella* and *Escherichia coli* share strikingly similar genomes, and the primary distinction between these bacteria arises not from the gain of novel genes in pathogenic *Shigella*, but rather from loss or inactivation of *E. coli* genes (273; 576). Gene content and synteny between these bacteria are highly conserved, with the *S. flexneri* 2a strain 2457T chromosome roughly 82% similar to the backbone of the laboratory-adapted *E. coli* K-12 strain MG1655 (576). In total, 3030 ORFs are shared between 2457T and MG1655, while only 175 ORFs are unique to *Shigella*; of these latter genes, most if not all were identified as members of the PAIs listed above. More strikingly, *Shigella* genomes have undergone excessive pseudogenization, as genes not required for the intracellular lifestyle were inactivated or deleted. At least 372 ORFs in *S. flexneri* 2457T have been classified as pseudogenes (576). However, these annotations only identify genes with remnants still remaining in the genome and do not take into account genes that may have been completely deleted. In a study by Wei *et al.* (2003), ortholog comparisons between 2457T and *E. coli* MG1655 proteins identified 879 total genes were either inactivated or absent in 2457T compared to MG1655, indicating that roughly 500 genes are absent in 2457T compared to MG1655 (576). Although we cannot be certain that these MG1655-specific genes were once present in 2457T, there are indications that at least some of these operons did exist in the ancestral strain(s) of *Shigella*. Compared to *E. coli* K-12, the genome of *S. flexneri* 2457T has undergone 15 inversions or translocations of regions greater than 5 kb, probably mediated by IS elements found at many of the inversion ends

(576). Pseudogene remnants surrounding these loci offer clues to which regions (and therefore, which genes) were disrupted or lost in the process of rearrangement. For example, a large deletion (~90 kb) containing part of the *cad* locus of *S. flexneri* 2a was previously described, with only fragments of the region (including a portion of *cadB*) remaining to indicate that the locus had probably once been present (122; 342). Sequence analysis of the genes downstream of the *cadB* fragment revealed the presence of several IS elements as well as the gene *ytfA* (122). In *E. coli* K-12, *ytfA* localizes to a different region of the chromosome rather than next to the *cad* operon. After *ytfA*, gene synteny between *E. coli* K-12 and *S. flexneri* 2a is reestablished, indicating that a large genomic rearrangement led to the loss of most of the *cad* operon in *S. flexneri* 2a.

While genes unnecessary to the novel niche of *Shigella* may have been pruned from the genome as a result of neutral selection, negative selection of genes inhibitory to the pathogenic lifestyle (i.e., antivirulence genes) has also occurred. As discussed previously, notable antivirulence genes (AVGs) in *Shigella* include *cadA*, encoding lysine decarboxylase; *nadA* and/or *nadB*, encoding catalytic enzymes in the NAD biosynthetic pathway; *speG*, encoding spermidine acetyltransferase; and *ompT*, which encodes an outer membrane permease (62). We hypothesized that additional AVGs have been lost or inactivated from *Shigella* isolates, and set out to identify novel AVGs in this organism.

Previous AVG work stems primarily from expressing candidate AVGs in wild type *Shigella* strains, and studying corresponding virulence phenotype effects. However, as explained in chapter four, putative AVGs can be difficult to identify, and certain biochemical pathways or structures, such as flagella and fimbriae, are complex systems to re-construct. Instead, we used an alternative approach by introducing the key virulence

factor, the *Shigella* T3SS, into commensal *E. coli* strains and then examining various virulence phenotypes. The *ipa/mxi/spa* operons of the *Shigella* T3SS were cloned onto the *Shigella* minimal invasion plasmid (*S*mip) under their native promoters, along with *virF* and *virB*, the transcriptional activators of the T3SS, and *icsA*, a gene required for actin-based motility (64). The *S*mip-IcsA (simply termed '*S*mip' for purposes of this chapter) was transformed into a plasmid-cured derivative (BS103) of our wild type *S. flexneri* 2a strain 2457T. Host cell invasion, intracellular growth, T3SS effector secretion, and cell-to-cell-spread of this strain were characterized in the previous chapter (64).

The *S*mip was introduced into four commensal strains of *E. coli*: SE11, ECOR07, ECOR47, and ECOR68. Host cell invasion, T3SS effector secretion and expression were analyzed in these strains and compared to BS103/*S*mip. Our data indicate that there are several inhibitors (potential AVGs) blocking virulence pathways in these *E. coli* strains.

METHODS AND MATERIALS

Strains

All strains (Table 5) were grown in Trypticase soy broth (TSB) or on TSB-CR plates with 1.5% agar and 0.25% Congo Red (Sigma). BS103 is the plasmid-cured derivative of the wild type *S. flexneri* 2a strain 2457T, while BS889 is BS103 carrying the Smip-*icsA* described previously (64). *E. coli* EPI300 was used to amplify the *S*mip-*icsA* construct to high copy number using the CopyControl™ induction solution as per the manufacturer's protocol (Epicentre). The commensal *E. coli* strains ECOR30, ECOR47, and ECOR68 are isolates from the original ECOR reference collection (399). SE11, a human commensal *E. coli* strain, was a gift from Masahira Hattori at the University of Tokyo (404). The pYD63 plasmid (*S*mip-*icsA*) was introduced into ECOR30, ECOR47,

Table 5. Strains and plasmids used in this study.

	Relevant genotype	**Source or reference**
Strains of *Shigella flexneri* 2a		
2457T	Wild type *S. flexneri* 2a	(181)
BS103	2457T cured of the virulence plasmid	(341)
BS889	BS103/pYD63	(64)
Strains of *Escherichia coli*		
ATM960	EPI300/pYD63	(64)
SE11	Wild type commensal *E. coli*	(404)
SE11/*S*mip	SE11/pYD63	This study
ECOR07	Wild type commensal *E. coli*	(399)
ECOR07/*S*mip	ECOR07/pYD63	This study
ECOR47	Wild type commensal *E. coli*	(399)
ECOR47/*S*mip	ECOR47/pYD63	This study
ECOR68	Wild type commensal *E. coli*	(399)
ECOR68/*S*mip	ECOR68/pYD63	This study
Plasmids		
pYD63	Smip-*icsA*	(64)

ECOR68, and SE11 by electroporation. In the strains carrying pYD63, kanamycin (50 μg/ml) and/or chloramphenicol (12.5 μg/ml) were added routinely to the growth media. HeLa cells were routinely grown in DMEM supplemented with 10% Fetal Bovine Serum (FBS) at 37°C in the presence of 5% CO_2.

Invasion assays

Gentamicin protection assays were performed as described in the previous chapter and elsewhere (224). Instead of calculating percent efficiency of invasion, however, data were expressed as bacteria recovered per monolayer normalized to an initial bacterial input of 1 x 10^8 cfu for each strain. To visualize internalized bacteria, infected monolayers were also stained with Giemsa as previously described, with a few modifications (472). Briefly, at 120 minutes post-infection, HeLa cells were washed with 1x PBS, and monolayers fixed with 2 mL glutaraldehyde/formaldehyde fixation solution (0.2% glutaraldehyde and 5.4% formaldehyde in 1x PBS) at 4°C for five minutes. Next, 2 mL Giemsa (1:20 dilution in dH_2O) was added to each well. After 1 hour staining, excess stain was aspirated and monolayers rinsed with distilled water, then air-dried and observed using light microscopy to identify infected cells.

Congo Red secretion assay

Congo Red secretion assays were performed as described in the previous chapter and elsewhere, with several minor modifications (610). Western blotting was performed with anti-IpaB monoclonal antibody (a gift from Edwin Oaks at the Walter Reed Army Institute of Research) at a dilution of 1:1000, and a secondary anti-mouse antibody conjugated to horseradish peroxidase (HRP) (Amersham Biosciences, 1:5000 dilution).

Blots were processed using the ImageQuantTM LAS 4000 according to manufacturer's instructions. Two separate chemiluminescent exposure times were carried out to detect the HRP enzyme: a brief primary exposure (10-100 seconds) followed by an additional, longer exposure (10 minutes) to ensure detection of weak bands.

qRT-PCR

Bacteria were grown in 5 mL TSB at 37°C to mid-exponential phase and bacterial input standardized to ~1 x 10^9 cells based on an OD_{600}. To lyse the bacteria, cells were pelleted and resuspended in 100 µL lysozyme solution (5 µL of 25 mg/mL lysozyme stock (Sigma) in 95 µL lysozyme buffer (50 mM Tris pH 8.0, 25 mM NaCl, 2 mM EDTA)). Cells were shaken at 1000 rpm for 10 minutes at room temperature, then 0.27 mM β-mercaptoethanol (Sigma) in 350 µL RLT buffer (Qiagen RNeasy kit) were added, and samples shaken for an additional 15 minutes to complete cell lysis. RNA isolation, cDNA synthesis, and qRT-PCR analyses were performed as previously described, with several modifications (89). RNA was then extracted using the Qiagen RNeasy kit (Qiagen) according to manufacturer's instructions. Total RNA (500 ng) was used to prepare cDNA using the QuantiText Reverse Transcriptase kit (Qiagen), and a control lacking reverse transcriptase was included for each cDNA synthesis reaction. Rotor-Gene SYBR green reactions (Rotor-Gene SYBR Green PCR kit, Qiagen) were set up in 25 µL reactions which included: 2 µL of template cDNA or the control lacking reverse transcriptase; 1x Rotor-Gene SYBR Green PCR master mix (Qiagen); and 1 µM each of transcript-specific primers corresponding to *virF*, *virB*, *ipaC*, or 16S genes (Table 6). A two-step qRT-PCR was performed on the Rotor-Gene Q instrument (Qiagen). An initial activation step (5 minutes at 95°C) was run, followed by forty total cycles of: 5 seconds

Table 6. Primers used in this study.

Primer Name	Sequence (5' to 3')	Reference
16S primers		
16S_RNAF2	CGTGCTACAATGGCGCATAC	This study
16S_RNAR2	TCACCGTGGCATTCTGATCC	
virF primers		
virF_RNAF3	AGATATAAAGGTTCGCTTGCATAAC	This study
virF_RNAR3	GCCCTTCATCGATAGTCAAAGT	
virB primers		
virB_RNAF1	CTGAAAGGCACGAGTGAC	This study
virB_RNAR1	GCATCCGAGAACTTGGTATTGG	
ipaC primers		
ipaC_RNAF2	ACACCCTTACTCCAGAGAACAC	This study
ipaC_RNAR2	GTGCGGAGAGCAGAAAGTAG	
virF gene primers		
virF_ampF	GGGCTTTATGCGTTCCGTATAG	This study
virF_ampR	CCGGGCTGTCATCATTAAACTG	
virF_seqF	CATTTCAACACTCCTATTCTGAGGAG	This study
virF_seqR	CAACAATCTTCCTTATCTGATC	
virB gene and promoter primers		
virB_ampF	GGCCAGTCACTCATATTCAC	This study
virB_ampR	CATCTATGGAGCTCTCAC	
virB_seqF	CGCTTGCTGCCTGAAAGGCACGAG	This study
virB_seqR	CTACTCTTGATGCCAGAAAACTAG	

at 95°C (denaturation) and 10 seconds at 60°C (combined annealing/extension). Flourescence was recorded after the combined annealing/extension step. Data for each transcript/strain were normalized to the 16S gene (ΔCT) and the $2^{(-\Delta\Delta Ct)}$ values were determined to calculate fold change in expression compared to BS103/*S*mip. Data are reported as the results of three independent experiments.

RESULTS

Invasion phenotypes of *E. coli*/*S*mip strains

Previous MLEE analysis by Pupo *et al.* (1997) suggested that *S. flexneri* 2a clusters most closely with several *E. coli* group D strains, specifically ECOR44 and ECOR47 (437). These strains were isolated from a healthy cougar and healthy sheep, respectively, as part of the *E. coli* Reference Collection (ECOR) (399). Because ECOR47 may share a closer evolutionary heritage with *S. flexneri* 2a than other commensal *E. coli* strains, it was utilized in this study. A second *E. coli* commensal strain, SE11, was isolated from a healthy adult human in Japan (404). SE11 falls into the B1 phylogenetic group, where strains of *Shigella* cluster based on previous phylogenetic analyses (437; 606). As an added benefit, this strain has been completely sequenced and annotated, and sequence analyses between SE11 and 2457T could be easily performed (404).

The *S*mip was transformed into SE11 and ECOR47, and host cell invasion, one of the primary virulence phenotypes of *Shigella*, was first examined (Figure 12). In a gentamicin protection assay, both SE11/*S*mip and ECOR47/*S*mip were severely attenuated for invasion compared to BS103/*S*mip; ECOR47/*S*mip and SE11/*S*mip invaded at approximately 2.35% and 0.25% of BS103/*S*mip, respectively. However, the number of bacteria recovered in the *S*mip-carrying strains was always higher than the

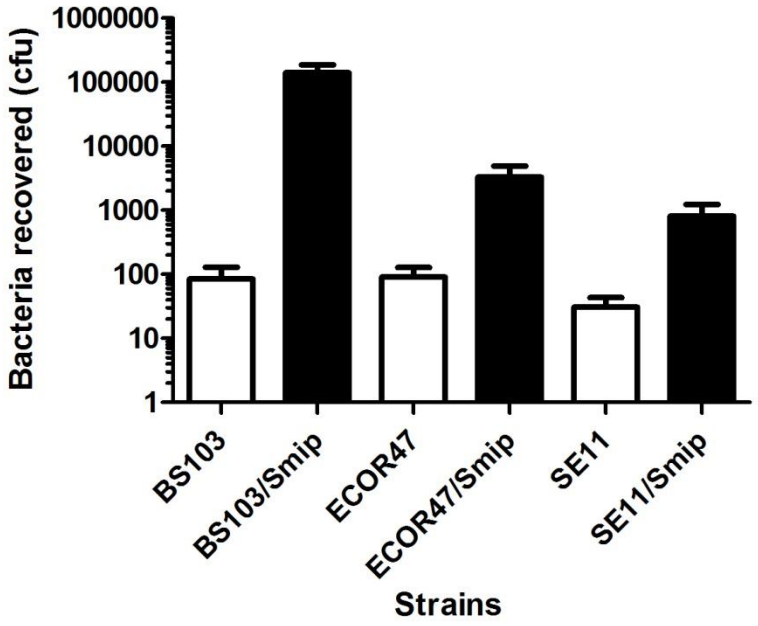

Figure 12. Invasion of *Shigella* and *E. coli* strains carrying the *S*mip.
A gentamicin protection assay was performed on HeLa cells infected with *S. flexneri* BS103/*S*mip, *E. coli* ECOR47/*S*mip, and *E. coli* SE11/*S*mip, and their respective parent strains (BS103, ECOR47, and SE11). The number of bacteria recovered (cfu/monolayer) was determined for each strain after normalizing bacterial input for each strain to 1×10^8 cfu/monolayer. Error bars represent the mean ± one standard deviation over three independent experiments.

number of bacteria recovered from the respective wild type *E. coli* parent alone. Although this value was not statistically significant for the *E. coli*/*S*mip strains, the trend remained consistent over three independent experiments.

To determine if the *E. coli*/*S*mip bacteria were able to invade the host cell, albeit very poorly, we stained infected monolayers with Giemsa to visualize the host cells (Figure 13). SE11/*S*mip and ECOR47/*S*mip were able to invade, as indicated by the presence of bacteria enclosed in an endosome-like structure, similar to what is seen during BS103/*S*mip invasion. Additionally, several bacteria lack this distinctive halo, indicating that both *E. coli*/*S*mip strains may be able to lyse the phagosome and escape into the host cytoplasm. While BS103/Smip formed protrusions and clearly exhibited intercellular movement, no membrane protrusions were discovered in the *E. coli*/*S*mip-infected monolayers.

T3SS secretion of *E. coli*/*S*mip strains

The attenuated invasion abilities of the *E. coli*/*S*mip strains (Figure 12) indicated that there might be an inhibitor(s) of invasion in the ECOR47 and SE11 backgrounds. In an attempt to determine where this inhibitor(s) was acting, the T3SS secretion ability of these strains was subsequently analyzed. As a prerequisite for invasion, the T3SS apparatus must be assembled at the surface of the bacterium, and the effectors essential for invasion, such as IpaB and IpaC, must be recruited to and secreted out of the needle (41; 241). A defect in secretion, therefore, would impair invasion. A Congo Red secretion assay was performed to access T3SS secretion of the invasin IpaB in the *E. coli*/*S*mip backgrounds (Figure 14). Preliminary work suggested that secretion of IpaB may be moderately attenuated in ECOR47/Smip; when the amount of secreted IpaB in

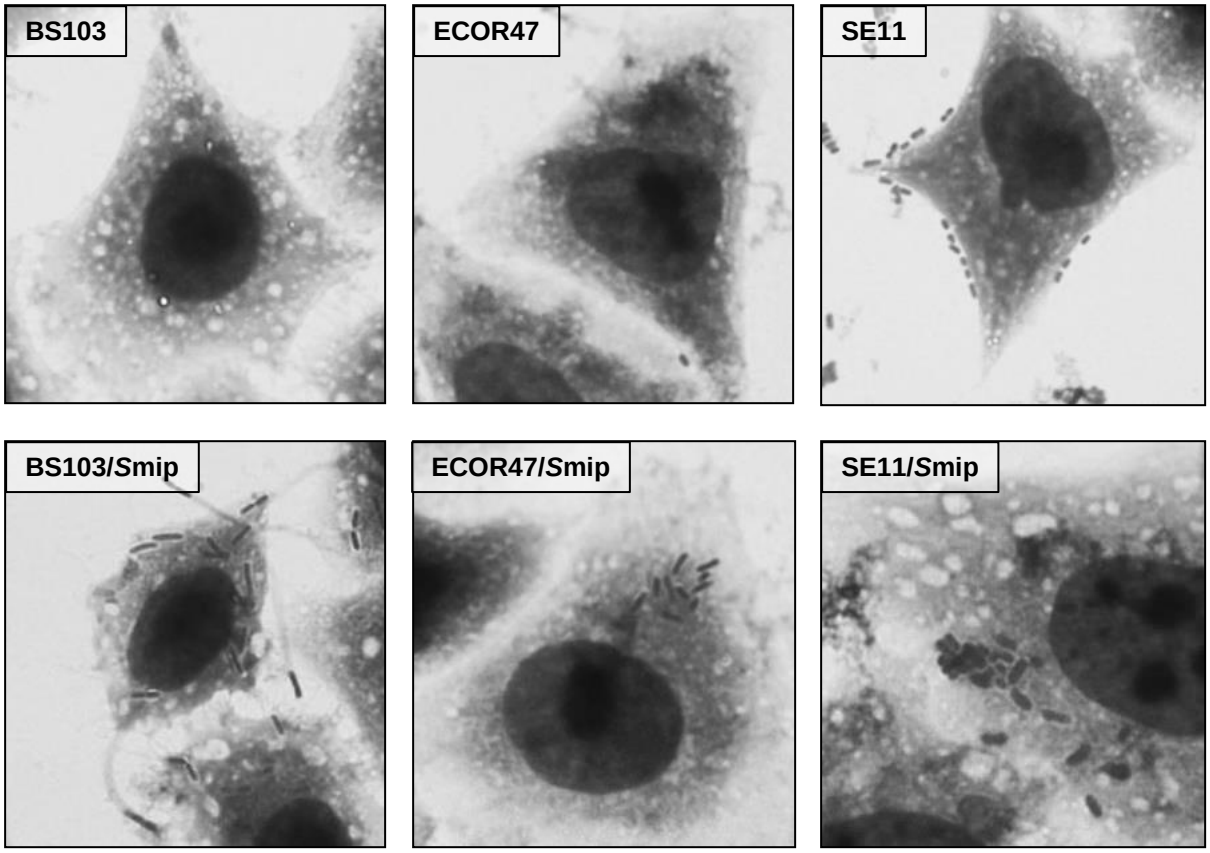

Figure 13. Visualization of HeLa cell monolayers infected with *Shigella* and *E. coli* strains carrying the *S*mip.
Giemsa-stained HeLa cell monolayers after 2 hours infection with BS103/*S*mip, ECOR47/*S*mip, SE11/*S*mip, and their respective parent strains (BS103, ECOR47, and SE11). Panels are representative of infected cells visualized in three independent experiments.

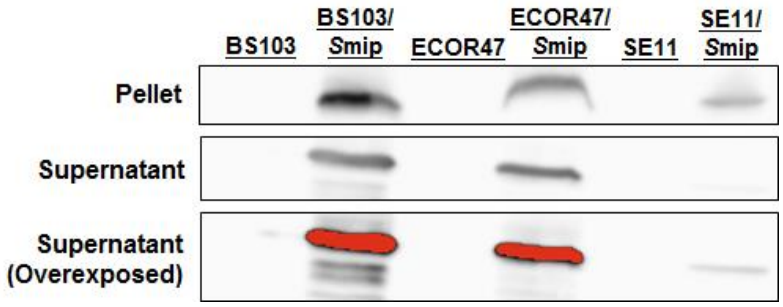

Figure 14. IpaB secretion from *Shigella* and *E. coli* strains carrying the *S*mip. A Congo Red secretion assay was performed on BS103/*S*mip, ECOR47/*S*mip, SE11/*S*mip, and their representative parent strains (BS103, ECOR47, and SE11). Western blot analysis of the IpaB invasion was performed on whole cell lysates (pellet) and secreted (supernatant) fractions. These blots are representative of three independent experiments.

BS103/*S*mip was normalized to 100%, only 82.8% of IpaB was present in ECOR47/Smip supernatants. Moreover, total IpaB is also lower in this strain (roughly 66.7% of total IpaB from BS103/*S*mip). However, the more surprising result was the dramatic reduction of total IpaB protein in SE11/*S*mip (16.1% of total IpaB from BS103/*S*mip), suggesting that protein expression or stability is severely compromised in this background. These data could indicate that a second T3SS inhibitor may be present in SE11 and absent in ECOR47. Alternatively, this inhibitor may be present in both strains, but more highly expressed or active in SE11 than ECOR47.

T3SS transcript analysis of *E. coli*/*S*mip strains

To explore the severe reduction of IpaB in the SE11/*S*mip strain, we sought to identify whether the block in protein expression occurred at the level of *ipaB* gene expression. The *ipa* operon (encoding *ipaBCDA*, *ipgCBA*, *icsB*, and *acp*) is the target of a temperature-dependent regulatory cascade to turn on the *Shigella* T3SS genes, so the genes involved in this regulatory cascade were examined (136). VirF is the primary transcriptional activator of the T3SS; upon expression, VirF binds to and activates the *virB* promoter, which encodes a second transcriptional activator (540). VirB, in turn, binds to and activates the *ipa* promoter (572). Relative levels of *virF*, *virB*, and *ipaC* transcripts were assessed in the ECOR47/*S*mip and SE11/*S*mip strains compared to BS103/*S*mip (Figure 15). A slight, but significant 4-fold decrease in *virF* transcript was seen in the ECOR47/*S*mip strain. Although *virB* and *ipaC* transcripts were consistently lower in ECOR47/*S*mip than BS103/*S*mip, the difference was not statistically significant. In SE11/*S*mip, however, the transcripts of all three genes were significantly decreased, with *virF*, *virB*, and *ipaC* demonstrating 4-, 17-, and 23-fold reductions, respectively. The

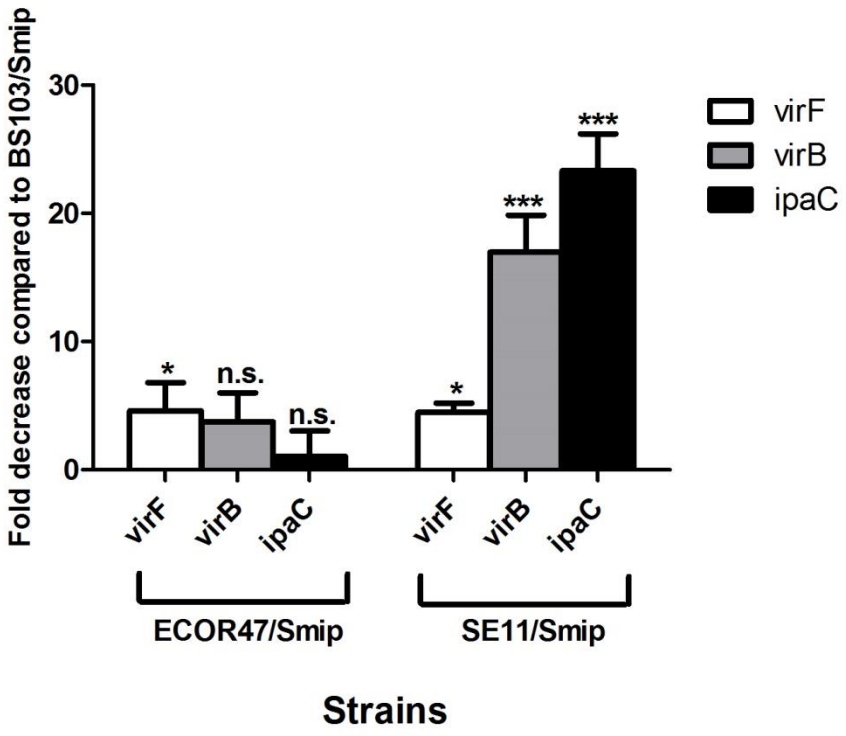

Figure 15. qRT-PCR analysis of T3SS transcripts.
Relative transcript levels of *virF*, *virB*, and *ipaC* in ECOR47/*S*mip and SE11/*S*mip comared to BS103/*S*mip. Each transcript was normalized to the 16S gene, and the $2^{(-\Delta\Delta Ct)}$ values determined to calculate relative gene expression. Error bars represent the mean ± one standard deviation over three independent experiments. * $p < 0.05$; *** $p < 0.001$; n.s. = not significant.

drastic reduction in gene transcript of the upstream transcriptional regulator, VirB, is most likely responsible for the severe decrease in IpaB protein demonstrated in the SE11/*S*mip strain. To ensure that spontaneous mutations in the *virF* gene or the promoter of *virB* were not responsible for this phenotype, these regions were amplified using primers virF_ampF/virF_ampR (*virF* gene) and virB_ampF/virB_ampR (*virB* gene and promoter region) (Table 6). The resulting PCR products were then sequenced using primers virF_seqF/virF_seqR (*virF* gene) and virB_seqF/virB_seqR (*virB* gene and promoter region) to ensure full coverage of these regions (Table 6). No mutations were detected in either the *virF* gene or the *virB* promoter in SE11/*S*mip. Therefore, an inhibitor of *virB* expression likely exists in this strain.

We attempted to identify the gene(s) involved in *virB* repression in the SE11/*S*mip strain by developing a transposon mutant library screen for restoration of *virB* expression. Briefly, the *virB* promoter was fused to a *lacZ* gene in the plasmid pRS415 (500), and the resulting construct transformed into SE11/*S*mip. For reasons unknown, growth of the resulting strain was severely attenuated, precluding use of this strain in a transposon mutant library analysis (data not shown).

Invasion phenotypes of additional *E. coli*/*S*mip strains

Although we were unable to identify the gene(s) involved in *virB* repression, we nevertheless wanted to determine if the putative inhibitor(s) present in the SE11 background was also expressed in other commensal *E. coli* strains. One of the defining features of an AVG is its presence and functionality in a majority of strains within the ancestral species; otherwise, in the absence of pseudogene remnants, it is impossible to determine if the current pathogen ever had, and then subsequently lost, the AVG (62). If

the defect in *virB* expression persists in additional *E. coli* strains carrying the *S*mip, this would be indicative of an AVG(s) rather than a unique suppressor in an outlier strain.

The *S*mip was introduced into two additional commensal *E. coli* isolates, ECOR07 and ECOR68, which were isolated from a healthy orangutan and giraffe, respectively (399). Gentamicin protection assays were performed to investigate the invasive ability of these strains (Figure 16). Similar to ECOR47/*S*mip and SE11/*S*mip, both ECOR07/*S*mip and ECOR68/*S*mip were severely attenuated for the invasive phenotype. To study T3SS gene expression, two qRT-PCR experiments were completed. Results demonstrated mild reduction of *virB* transcript in ECOR07/Smip (a 3.1-fold decrease in the first trial, and a 3.5-fold decrease in the second) (data not shown). In ECOR68/*S*mip, there was a significant reduction of *virF* transcript (33-fold and 31-fold decreases, respectively), and no detectable *virB* transcript in either trial (data not shown). Due to the block in expression of the upstream regulator, *virF*, we were unable to determine if a separate, additional inhibition occurred at the level of *virB* expression in ECOR68/*S*mip.

DISCUSSION

In this study, we introduced the *Shigella* minimally invasive plasmid (*S*mip) into several commensal *E. coli* isolates and studied the virulence phenotypes of the resulting strains. All *E. coli*/*S*mip strains were highly attenuated for invasion, although the presence of invaded cells in infected monolayers suggest that these strains do invade, albeit minimally. As we will discuss later, we cannot rule out the possibility that a chromosomal gene(s) present in *Shigella*, but absent in commensal *E. coli*, is responsible for the differences in these phenotypes, so we will present two separate hypotheses: first,

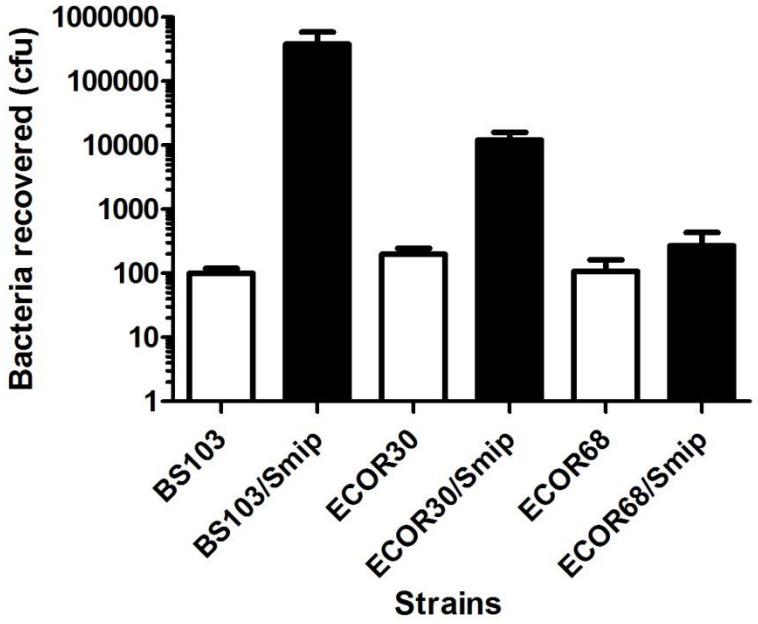

Figure 16. Invasion of *Shigella* and *E. coli* strains carrying the *S*mip.
A gentamicin protection assay was performed on HeLa cells infected with *S. flexneri* BS103/*S*mip, *E. coli* ECOR07/*S*mip, and *E. coli* ECOR68/*S*mip, and their respective parent strains (BS103, ECOR07, and ECOR68). The number of bacteria recovered (cfu/monolayer) was determined for each strain after normalizing bacterial input for each strain to 1 x 10^8 cfu/monolayer. Error bars represent mean ± one standard deviation over three independent experiments.

that gene loss or inactivation is responsible for the ability of BS103/*S*mip to invade epithelial cells; and second, that gene gain is responsible.

In support of the first hypothesis, the majority of *Shigella* virulence factors are either encoded on the large virulence plasmid present in all *Shigella*, or restricted to several chromosomal pathogenicity islands (PAIs), none of which are universal to all *Shigella* strains (340; 480). Furthermore, none of these PAIs appear to play a role in host cell entry (16; 214; 259; 328; 344; 371; 440; 566). In the previous study comparing *Shigella* and *E. coli* genomes, Wei *et al*. (2013) found that only 175 ORFs were unique in *Shigella* compared to *E. coli* K-12 MG1655 (576). This is probably an over-estimation, however. K-12 strains such as MG1655 have undergone considerable lab-adaptation through successive passaging and exposure to ultraviolet (UV) light, ionizing radiation, and a variety of mutagens; as a consequence of gene loss and inactivation, they have lost the ability to colonize the human gut (33; 78; 504). MG1655 itself has undergone UV radiation treatment, passaging on blood agar, and exposure to acridine orange (33). The chromosome of MG1655 carries approximately 4133 protein-coding genes, significantly fewer than a commensal such as SE11, which carries 5002 (404). *S. flexneri* 2a strain 2457T, meanwhile, harbors roughly 4075 protein-coding genes on its chromosome (BioCyc, 03/24/15, (95)). In summary, the laboratory-adapted MG1655 is a poor comparison strain to utilize for studying gene loss and inactivation in *Shigella*, as it does not constitute a true wild type commensal *E. coli* strain that would more closely resemble the ancestors that probably gave rise to *Shigella* (308). .

None of the known AVGs, when expressed in wild type *Shigella* strains, inhibit cell entry (39; 171; 342; 346; 381; 434). Although the exogenous addition of quinolinic

acid significantly attenuates *Shigella* invasion, the expression of functional *E. coli nadA*/*nadB* genes in a wild type *Shigella* background does not (Maurelli lab, unpublished data), suggesting that expression of these genes alone is not sufficient for attenuation. Therefore, the poor invasiveness of the *E. coli*/*S*mip strains in this study may demonstrate the presence of at least one novel AVG(s) in these commensal *E. coli* backgrounds.

To further investigate the invasion phenotype differences between BS103/*S*mip and the *E. coli*/*S*mip strains, we evaluated T3SS secretion and expression. Variable expression and secretion defects in the *E. coli*/*S*mip suggest that there may be multiple blocks hampering the full secretion or expression phenotypes depending upon the strain background. In the secretion assay, ECOR47/*S*mip only exhibited moderate decreases in secretion and expression of the T3SS invasin IpaC, which does not correlate with the severe invasion defect of this strain, suggesting that there may be other factors preventing optimal invasion in this strain background. In contrast, SE11/*S*mip exhibited a significant decrease in total IpaC, which was subsequently tracked to an expression defect of the upstream T3SS regulator gene, *virB*. A separate T3SS expression defect in the regulator *virF* blocked downstream expression in ECOR68/*S*mip. From these data, we concluded that there may be at least three potential AVGs: one at the level of *virF* expression in ECOR68/*S*mip; one at the level of *virB* expression in SE11/*S*mip; and a final putative AVG to account for the reduced invasion phenotype of ECOR47/*S*mip.

The nature of these AVGs or suppressors is yet unclear, though we can suggest a few possibilities. The T3SS expression defects in SE11/*S*mip (at the level of *virB*) and ECOR68/*S*mip (at the level of *virF*) may be due to one or more of the following: regulators/proteins which bind to the promoters of these genes to block transcriptional

activation; proteins that bind to and stabilize the repression activity of the H-NS repressor; or proteins that bind to and catalytically silence the upstream regulators (VirF or FIS, respectively). In regard to the strains that did not have significant expression defects (ECOR47/*S*mip and ECOR07/*S*mip), yet still exhibited attenuation in invasion, there are several possible explanations. First, although we examined *ipa* operon transcription, we did not include the *mxi* or *spa* operons also necessary for assembly of the T3SS needle. Repression at either the *mxi* or *spa* operon promoters would preclude T3SS assembly and therefore invasion. Conversely, if the T3SS needle is appropriately assembled in these strains, the needle itself could be obstructed by surface structures that commensal *E. coli* strains typically express and the *Shigella* species lack. Although we do not yet have detailed sequence analyses for ECOR06, ECOR68, or ECOR47, SE11 has been fully sequenced and annotated, and expresses fimbriae, a flagellum, and curli, which are all absent from *S. flexneri* 2a strain 2457T (404; 576). These surface structures could block or hinder the ability of the T3SS needle to make adequate contact with the host cell in order to invade. Exploration of this latter hypothesis is complicated, however, as many of these systems encompass a wide variety of structural genes and regulators, and reconstruction of these systems into *Shigella* would be difficult. Conversely, if more than one structure is involved in blocking the T3SS needle, the loss of a single system may not necessarily restore invasion in the *E. coli*/*S*mip strains.

Thus far, our speculation carries the assumption that gene loss or inactivation of commensal *E. coli* gene(s) would allow for the restoration of expression and invasion phenotypes in the *E. coli*/*S*mip strains. However, there are two valid alternative hypotheses: firstly, that a novel gene(s) required for optimal invasion was laterally

transferred onto the *Shigella* chromosome; or secondly, that a gene(s) shared between commensal *E. coli* and *Shigella* succumbed to a mutation(s) to allow for the invasive phenotype, leaving *Shigella* with either an altered or differentially expressed gene. If an essential bacterial gene inhibited virulence, for example, a mutation in this gene which increased virulence but did not disable the gene's essential function would be beneficial. Conversely, an inhibitor of virulence could be temporally regulated so it is never expressed when the *Shigella* T3SS is active. These variants would be more difficult to identify compared to pseudogenized genes, and would require either the expression of a *E. coli* library in a *Shigella* strain or intense full genome comparisons between multiple *Shigella* and commensal *E. coli* strains. Such an adaptation would still be interesting to pinpoint, however, as it could demonstrate an example of functional alteration of an existing gene rather than complete loss or inactivation. Given our current definition of antivirulence, this would not constitute an AVG, but perhaps the AVG definition must be broadened to cover such a hypothetical event (62).

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CHAPTER 7: *CHLAMYDIA* EVOLUTION AND VIRULENCE

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The literature review presented here is intended to familiarize the reader with the bacterial pathogen Chlamydia *to provide an introduction for Chapter VIII. Currently, this chapter has not been submitted for publication.*

K. A. Bliven and A. T. Maurelli both contributed ideas for the general outline of the chapter. K. A. Bliven reviewed the literature and wrote the chapter.

Note: The figure and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

Members of the genus *Chlamydia* are Gram-negative, obligate intracellular bacteria that invade eukaryotic host cells of humans and animals. *Chlamydia* infections are responsible for a wide range of maladies, including sexually-transmitted disease (STD), trachoma, pneumonia, and spontaneous abortion. This bacterium possesses a unique biphasic lifestyle transitioning between elementary bodies, the compact, extracellular, and infectious form, and reticulate bodies, the larger, intracellular, metabolically active form. Although a lack of efficient genetic tools has slowed progress on much of the basic biology of this organism, several genes or gene systems have been implicated in virulence, including a type three secretion system and the cytotoxin-encoding *tox*. Other factors, such as *ompA*, the polymorphic membrane proteins (Pmps), and genes located within the *Chlamydia* plasticity zone (PZ) may contribute to host or tissue tropism between serovars or species. In addition to sequence variation between species, species-specific gene inactivation/deletion events are indicative of a robust evolutionary history, which has produced a genus of highly specialized, niche-adapted pathogens.

INTRODUCTION

Infections with *Chlamydia*, a genus of Gram-negative, obligate intracellular bacteria, present substantial healthcare and economic challenges worldwide. *Chlamydia trachomatis* is the most commonly reported sexually-transmitted disease (STD) in the United States, with almost 1.5 million cases reported to the Centers for Disease Control and Prevention in 2013 (99). Infection with an ocular serovar of *C. trachomatis* can produce trachoma, the leading cause of preventable infectious blindness worldwide; the

World Health Organization estimates that of the approximately 2.2 million people per year who become infected, 1.2 million will eventually lose their sight (588). Another *Chlamydia* species, *C. pneumoniae*, is a common cause of infectious pneumonia worldwide (209). In addition to the considerable healthcare costs involved in treating infected humans, the economic loss from Chlamydial infections in animals is severe: in the United Kingdom alone, where *C. abortus* is most prevalent, costs due to enzootic abortion in sheep are estimated at around £20 million per year (approximately $30 million U.S) (324).

All members of the *Chlamydia* genus share a unique biphasic lifestyle, transitioning from elementary bodies (EBs), the extracellular form that is highly compact, infectious, and almost entirely metabolically dormant, to reticulate bodies (RBs), the intracellular form that is larger, non-infectious, and capable of metabolic activity and replication within the host (372). The genus itself is divided into nine species: *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, *C. suis*, *C. muridarum*, *C. avium*, *C. caviae*, *C. felis*, *C. abortus*, and *C. pecorum* (158). Certain *Chlamydia* species have a fairly limited host range: *C. trachomatis* is a human-specific pathogen; *C. suis* infects swine; guinea pigs can acquire *C. caviae* infections; and *C. muridarum* is a disease of mice and hamsters. Other *Chlamydia* species have a much broader host range, and many are zoonotic (580). *C. pneumoniae* strains fall into one of three separate biovars, depending on their primary host: human, koala, and equine (159). *C. felis* can be spread from domestic cats to humans. *C. abortus* is a primary cause of spontaneous abortion in sheep, but humans in close contact with infected ruminants also risk acquiring the infection. *C. psittaci*, so named for its prevalence in psittacine birds such as parrots, can infect

humans, horses, cattle, and tortoises. Lastly, *C. pecorum* has been isolated from numerous mammal species, including cattle, sheep, goats, koalas, and swine. More recently, atypical strains of *Chlamydia* have also been reported that may represent additional species in novel hosts, including *C. gallinacea* in poultry and *C. ibidis* in the African Sacred Ibis bird (466; 567).

Chlamydia species are further differentiated into serovars based on serological reactions to the major outer membrane protein (MOMP) encoded by *ompA* (82). In *C. trachomatis*, at least nineteen different serovars (A, B, Ba, C, D, Da, E, F, G, H, I, Ia, J, Ja, K, L1, L2, L2a, and L3) have been identified. Serovars A-C are responsible for the chronic ocular disease trachoma, while serovars D-K result in lower or upper genital tract infection (359). The L serovars, known as the lymphogranuloma venereum (LGV) serotypes, also cause genital tract infections, but are associated with more severe, invasive disease, and can result in complications such as genital ulcers and proctitis (331). Although six different serovars have also been identified in *C. psittaci*, many species of *Chlamydia* either consist of only one serotype (*C. felis*), or have not been characterized enough to distinguish novel serovars (105).

A review on *Chlamydia* is not complete without mention of the genetic tractability (or lack thereof) of this organism. Until recently, the genetic limitations of working with this obligate intracellular organism made genetic manipulation of *Chlamydia* extremely difficult. Previously, *Chlamydia* could not be cultured outside of the host cell, transformation or transduction of *Chlamydia* was largely unsuccessful, and plasmid maintenance once inside the bacterium was unstable (270). The gains made thus far, therefore, are a tribute to the scientists who have struggled to understand this

pathogen in the absence of an effective system for site-directed gene deletion or complementation. Instead, surrogate bacterial systems, inhibitors, and chemical mutagenesis screens were common tools employed to study this pathogen. Within the last few years, genetic systems to efficiently transform *Chlamydia* are finally starting to emerge, and an axenic medium has been developed to keep the bacteria metabolically active outside the host for several hours (403; 571). The next decade should be an exciting and busy time for the field.

In order to set the background for the arginine decarboxylase manuscript, this chapter will present an overview of *Chlamydia* speciation and outline the factors hypothesized to be important for the host and tissue tropism of this pathogen. To parallel the previous *Shigella* pathogenesis chapter, the known virulence factors of *Chlamydia* will also be briefly discussed.

THE EVOLUTIONARY HISTORY OF *CHLAMYDIA*

The divergence of separate sister groups from a common ancestor in the order *Chlamydiales* is postulated to have occurred around 700 million years ago, resulting in environmental endosymbionts that thrive within free-living amoebae (*Parachlamydiae*) and the pathogenic chlamydiae (*Chlamydiaceae*) (251; 393). Other related families belonging to the *Chlamydiales* order include the pathogens *Waddliaceae* and *Simkaniaceae*, although their evolutionary timescales related to *Chlamydiaceae* are currently unknown (159; 462). Curiously, the family *Chlamydiaceae* is also hypothesized to share an evolutionary heritage with the cyanobacterial endosymbiont ancestor of the modern plant chloroplast. Such ancestry would account for the presence of

Chlamydiaceae genes with high sequence similarity to plant genes, such as the ATP/ADP translocase (77).

The family *Chlamydiaceae* consists of a single genus, *Chlamydia* (Figure 17). In terms of phylogeny, there appear to be two main clusters of *Chlamydia* species: *C. caviae*, *C. felis*, *C. abortus*, and *C. psittaci* group closely together in the so-called *C. psittaci* group while *C. trachomatis* and *C. muridarum* form a second cluster termed the *C. trachomatis* group. *C. pneumoniae* and *C. pecorum*, in contrast, are independently divergent from these two main clusters (393). The first full genome sequence of a *C. suis* strain was not released until after the Nunes and Gomes manuscript was accepted, and therefore was not included in the original phylogenetic analysis (133). However, sequence analyses of the chlamydial plasticity zone (PZ), where the *Chlamydia* genomes demonstrate the majority of interspecies variation, indicates that *C. suis* likely clusters with *C. trachomatis* and *C. muridarum* in the *C. trachomatis* group. Concomitant with the emergence of modern human lineages, the ancestor of *C. trachomatis* probably branched from the other *Chlamydia* species in the last 6 million years (393). The time frame for *C. muridarum* and *C. suis* divergence from *C. trachomatis* is yet uncertain.

Like most obligate intracellular organisms, both *Parachlamydiae* and *Chlamydiaceae* have experienced significant genome reduction, as genes unnecessary for survival inside the host were inactivated and discarded, and resources were more efficiently parasitized from the eukaryotic host. Although *Parachlamydiae* and *Chlamydiaceae* share around 700 genes, the environmental chlamydiae have larger genomes (~2.5 Mb) than the pathogenic chlamydiae (~1 Mb), probably due to more extensive variations in environmental conditions within the amoeba (251). In contrast to

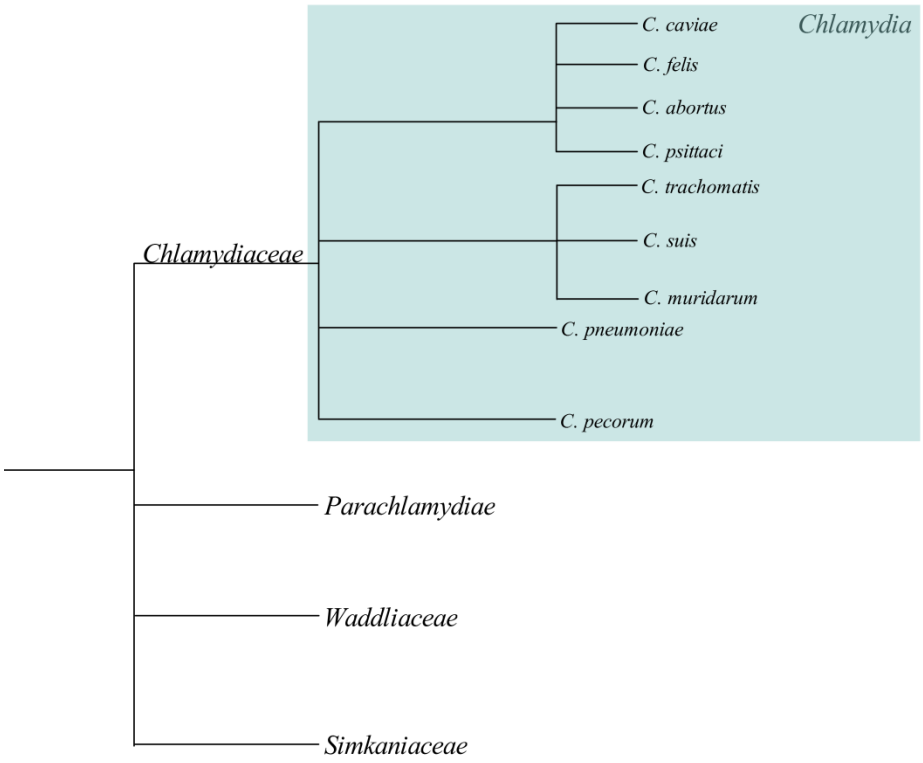

Figure 17. Proposed phylogenetic tree for the *Chlamydiales*.
Species within the *Chlamydia* genus are blocked in green. Branch length does not correlate with any proposed evolutionary timescale, as many of these divergence events have not yet been studied with respect to time.

the pathogenic chlamyidae, the environmental chlamydiae encode a complete tricarboxylic acid (TCA) cycle, while *Chlamydia* is missing three key enzymes (citrate synthase (*gltA*), aconitase (*acnB*), and isocitrate dehydrogenase (*icd*)) (251; 304). Either unidentified enzymes perform these functions in *Chlamydia*, or the pathogen bypasses the need for these enzymes entirely, although enzymes involved in such a bypass have not yet been recognized in *Chlamydia* (513). Furthermore, the *Parachlamydiae* encode a type four secretion system (T4SS), which may be important for interactions with the amoebal host; interestingly, the pathogenicity island harboring the T4SS appears to be one of the only post-acquisition events in the environmental chlamydiae after divergence from the pathogenic chlamydiae (251). The genomes from both environmental and pathogenic chlamydiae appear to have stabilized in their respective niches, however, as there have been few pseudogenes identified in either genus (251). This stabilization, coupled with the relatively small genome size, is an indication that the current preserved genes are likely either essential or advantageous for the lifestyle of these organisms. Additionally, the low frequency of pseudogenes or insertion elements suggests that the reductive evolution of *Chlamydia*/*Parachlamydiae* was not a recent evolutionary event. To make matters more interesting, the narrow niche specialization of *Chlamydia* has resulted in not only elimination of many redundant genes, but emergence of enzymes with dual functions and/or broad specificity (8; 57; 296).

Horizontal gene transfer (HGT) between *Chlamydia* and other organisms is thought to be a rare occurrence based on whole genome sequence analyses (270). The exact reason for this is unclear, but it likely involves the obligate intracellular nature of the bacterium. Several layers of membrane, including those of the eukaryotic host, the

inclusion and bacterium itself, separate the metabolically active RB from the environment. Extracellular EBs, largely metabolically inert, with histone-like proteins that bind DNA sequences, may not be able to efficiently acquire or incorporate DNA (40; 222; 270). Furthermore, phages that infect *Chlamydia* are rare, and therefore transduction events likely do not contribute significantly to HGT in this pathogen. Currently, no phages are known to infect *C. trachomatis*, and only five phages (Chp1, ΦCPG1, Chp2, ΨCPAR39, and Ch3) have been identified that infect other *Chlamydia* species (160; 256; 322; 455). As a result, *Chlamydia* is thought to have evolved largely in isolation, reflected by genomes that have a high degree of synteny (gene order) and an invariable G+C content (448). However, genetic recombination within the genus between serovars or even species is suspected based on whole genome sequence analyses, and genetic recombinants can be generated *in vitro* during mixed infections (128; 202; 521). The precise mechanism for this transfer is not yet clear.

HOST AND TISSUE TROPISM OF *CHLAMYDIA*

Studies on genetic variability between the *Chlamydia* species are slowly teasing out genes that may be responsible for host and tissue tropism. Although the majority of the *Chlamydia* genomes are surprisingly similar, both in content and gene order, there is a unique hypervariable region near the replication terminus of the genome that is thought to play a significant role in different virulence phenotypes (393). Termed the plasticity zone (PZ), this region encompasses genes including a cytotoxin; the *guaAB-add* cluster; the membrane attack complex/perforin (MACPF) domain gene; the phospholipase D (PLD) enzyme family; and the tryptophan operon (380; 393). The region itself varies in size and content between genomes, ranging from 18-81 kB (380).

The cytotoxin-encoding gene, *tox*, is conserved in most strains of *C. muridarum*, *C. caviae*, *C. psittaci*, *C. felis*, and *C. pecorum*; in fact, *C. pecorum* and *C. muridarum* contain two and three copies of the gene, respectively (380). In contrast, the majority of *C. pneumoniae*, *C. abortus*, and *C. trachomatis* strains no longer produce functional cytotoxin; the exception is *C. trachomatis* serovar D, which encodes a truncated, but still functional, cytotoxin (448; 495). Several strains of *C. psittaci* also carry deletions or frameshifts in *tox*, although corresponding effects on functionality have not yet been studied (449). *tox* appears to be homologous to the large clostridial cytotoxins produced by *Clostridium difficile*, which function as glucosyltransferases against eukaryotic Rho GTPases, ultimately disrupting the host actin cytoskeleton (46; 80). The cytotoxity seen in epithelial cells infected with *C. trachomatis* serovar D and *C. muridarum*, therefore, may be at least partially attributable to the protein encoded by this gene (46). Furthermore, a hypervirulent clinical strain of *C. trachomatis* serovar L2, which sequence analysis suggests had undergone recombination with a strain of *C. trachomatis* serovar D, had acquired the truncated *tox* gene (506). Although experimental confirmation would be required to confirm the importance of the *tox* gene in the hypervirulence phenotype of this strain, this isolate is highly cytotoxic in cell culture, in contrast to other clinical L2 isolates.

The differential activity of the tryptophan operon (*trpRBA*) between serovars of *C. trachomatis* is one of the earliest examples of a gene system associated with tissue tropism in *Chlamydia* (85). In most bacteria, this system is important in the biosynthesis of tryptophan from the precursor chorismate, and consists of the enzymes TrpE/G, an anthranilate synthase; TrpD, an anthranilate phosphoribosyl transferase; TrpF, an

anthranilate phosphoribosyl isomerase; TrpC, an indole-glycerol phosphate synthase; and TrpA/B, a tryptophan synthase tetramer that can convert indole-3-glycerol phosphate to tryptophan. In the genital serovars of *C. trachomatis* (D-K), however, the tryptophan operon consists of only the synthase genes (*trpA*/*trpB*) and the repressor gene, *trpR* (85; 357). *Chlamydia* lack known orthologues to several upstream enzymes in this biosynthetic pathway, including TrpE, G, D, and C. Phenotypically, all strains of *Chlamydia* are auxotrophic for tryptophan, indicating that they must import either tryptophan or the biosynthetic intermediate indole, which can be converted to tryptophan by tryptophan synthase (TrpA/B) (168). In *E. coli*, *mtr* encodes for a tryptophan transporter (235). A putative homolog of *mtr* is the *Chlamydia* gene *tyrP*, although its functionality has not yet been determined (15). Although no indole transporter has been identified, the conservation of functional tryptophan synthase genes in the genital serovars of *C. trachomatis* suggests that this operon still maintains an important function for the bacterium (85). Interferon-gamma (IFN-γ), a cytokine involved in the protective host response against *Chlamydia* infection, has been shown to deprive the pathogen of tryptophan by activating the L-tryptophan degradation enzyme indoleamine 2,3-dioxygenase (IDO). IDO depletes host cell stores of tryptophan and prevents *Chlamydia* replication (536). Therefore, conservation of these genes provides the bacterium an alternate source of tryptophan in an IFN-γ-rich environment such as the infected genital tract, as bacteria likely import the precursor indole and subsequently convert it to tryptophan (85; 265). Because addition of exogenous indole can rescue tryptophan-starved *Chlamydia*, it is speculated that the bacteria probably does have a transporter(s) for indole import (85). In contrast to the genital strains, the *C. trachomatis* ocular

serovars (A-C) have lost the functional tryptophan biosynthetic pathway, and presumably import tryptophan directly from the host cell. Inactivation of the tryptophan synthase system in the ocular strains is due to a single base pair frameshift mutation within *trpA*, which results in a truncated, nonfunctional protein. Although the exact reason for inactivation of the *trp* operon in these strains is not yet known, Caldwell *et al.* speculate that there may not be an exogenous source of indole within the sterile ocular environment, unlike the genital tract where indole is likely produced by the normal microbiota (85). Additionally, the immunoprivileged eye may lack the cells necessary to produce IFN-γ. *C. caviae* also has a complete tryptophan operon within the PZ; *C. felis* and *C. pecorum* carry the operon outside the PZ, which may suggest that functional tryptophan synthase is also critical within their respective niches (380). *C. muridarum*, *C. abortus*, and *C. pneumoniae* are missing the entire operon.

The remaining genes located within the PZ have not yet been fully characterized. The *Chlamydia* MACPF domain gene and the phospholipase D (PLD) enzyme family genes are homologous to genes that encode lipid-modification proteins (535). In the PZ, PLD enzyme family genes have only been identified in strains of *C. trachomatis*, *C. pecorum*, and *C. muridarum*. Although their exact roles in the lifestyle of *Chlamydia* remain unknown, they may be important for lipid modification of host cells (380; 385). In *C. trachomatis*, the MACPF domain gene, CT153, is expressed during the mid-developmental cycle in all serovars, and undergoes proteolytic processing. This processing may be required for function, as some MACPF proteins are only activated upon cleavage, although the uncleaved protein may have a separate, additional function (535; 555). *C. pecorum* and the koala strains of *C. pneumoniae* also appear to have full-

length MACPF domain genes (380). In isolates of *C. felis*, *C. muridarum*, and *C. caviae*, the MACPF domain gene is absent, while *C. abortus* and human-specific strains of *C. pneumoniae* appear to have truncated MACPF domain genes. *C. psittaci* encodes for two MACPF domain genes, one full-length and one truncated. Another set of genes, the *guaAB-add* cluster, encode enzymes important in the purine salvage pathway; these genes are absent or truncated in *C. trachomatis* and *C. abortus*, but present in *C. muridarum*, *C. felis*, and some *C. pneumoniae* and *C. psittaci* genomes (449). A number of hypothetical genes also sit in the PZ region of some, but not all, *Chlamydia* species, and their functionality and contribution to tissue or host tropism have not yet been defined.

Outside of the PZ, other genes may contribute to tissue tropism. Biotin, an important cofactor for enzymatic reactions, is an essential vitamin for all bacteria, and therefore must be biosynthesized or scavenged from the environment. All *Chlamydia* species, with the exception of *C. abortus*, encode members of a putative biotin biosynthesis system (*bioA*, *bioD*, *bioF*, *bioB*), although there is not yet any evidence that this system is functional (177). Scavenging is probably the preferred method of biotin acquisition in *Chlamydia*, however, as it would require less effort from the bacterium in biotin-rich environments. In most bacteria, the complete biotin transporter consists of three proteins: BioY, which binds the substrate and may function as the basic transporter; BioM, an ATPase; and BioN, an integral membrane protein (236). In *Rhodobacter capsulatus*, BioY alone demonstrated transport activity, albeit at a decreased efficiency to the complete BioYMN transporter (236). In *Chlamydia*, orthologs of *bioY*, but not *bioM* or *bioN*, have been identified (177). When the *C. trachomatis* BioY is expressed in a surrogate *Escherichia coli* system, it functions as both a high-affinity and high-capacity

transporter of biotin (177). Although there is no evidence that *bioM* or *bioN* existed in the *Chlamydia* genome at one time, Fisher *et al.* speculate that evolution of a single-protein high-affinity biotin transporter made these genes superfluous, and they were likely inactivated and eventually lost from the genome (177). The gene *bioY* is present and well-conserved in *C. trachomatis*, *C. abortus*, *C. caviae*, and *C. felis*, but absent from the genomes of *C. pneumoniae* isolates. Biotin is scarce in lung tissue, where *C. pneumoniae* invades, which would make expression of the transport pathway impractical, and presumably *C. pneumoniae* acquires biotin through the biosynthetic pathway (116; 177). Conversely, the biosynthetic biotin pathway in *C. abortus* appears to be degraded, and this species may only utilize the transport system, suggesting that the niche of *C. abortus* supplies the pathogen with exogenous biotin.

Additional genes that may influence tissue/host tropism include the major outer membrane protein (MOMP), encoded by *ompA*, and the polymorphic membrane protein genes (Pmps), which encode type V autotransporters (13; 44). Both of these will be discussed later in the adherence/invasion section.

THE TYPE THREE SECRETION SYSTEM (T3SS)

The T3SS is essential to the lifestyle of *Chlamydia*, and the ancestor of both the environmental and pathogenic chlamydiae likely acquired this system to invade early eukaryotes (251). As the genetic systems for *Chlamydia* have been limited, much of the work on the *Chlamydia* T3SS has been conducted in surrogate systems such as *Shigella* and *Yersinia* (520). Mutants of *Shigella* or *Yersinia* defective in T3SS structural genes are complemented with putative *Chlamydia* homologs to see if partial or full T3SS function can be restored, and putative *Chlamydia* effector proteins are expressed in wild type

Shigella or *Yersinia* strains to determine whether these effectors are substrates for T3SSs (102; 174; 175; 520).

The basic structure of the T3SS was described in the previous chapter on *Shigella* pathogenesis. Because our knowledge of the T3SS in *Chlamydia* has been considerably hampered from the lack of effective genetic tools, much less is known about T3SS function and effector interactions with the host. There are many obvious distinctions between the intracellular lifestyles of *Shigella* and *Chlamydia*, which could be attributed to differences in T3SS-mediated activity; for example, *Shigella* lyses the endosome shortly after invasion, while *Chlamydia* modifies the endosome and subverts host cell trafficking to the maturing inclusion (354). This section will present what is known about the T3SS in *Chlamydia*.

Genetics, organization, and regulation

The genetic organization of the *Chlamydia* T3SS is unique among Gram-negative bacteria. Instead of associating with a pathogenicity island (enteropathogenic *E. coli*) or a large plasmid (*Shigella*), the T3SS operons of *Chlamydia* are found scattered throughout the genome (237; 519). It is most probable that acquisition of the *Chlamydia* T3SS was a very ancient event, and the system has undergone extensive genetic rearrangements since. The most common indicator of lateral gene transfer is a lower G+C content within the transferred region compared to the remainder of the genome; however, this cannot be demonstrated in *Chlamydia*, as the G+C content of *Chlamydia* is already low (519). Despite this, there are some indications that the T3SS in *Chlamydia* was acquired. Several of the *Chlamydia* T3SS operons share high synteny with the T3SSs of *Shigella* and *Yersinia*. Additionally, although homologs to several of the basic structural

components of the *Shigella* T3SS have not yet been found in *Chlamydia* (MxiG, MxiH, MxiI, MxiK, Spa13, and Spa32), many more have (MxiA, MxiD, MxiJ, MxiN, Spa9, Spa24, Spa29, Spa33, Spa40, Spa47, and Spa40), suggesting that even though several of the *Chlamydia* T3SS genes may have undergone significant divergence, many of the main structural components are similar to those in known T3SSs, indicating a common system ancestry (4; 519).

At least four separate loci spread throughout the *Chlamydia* genome are thought to contain genes necessary for assembly of the T3SS (519). Within these loci, the T3SS is postulated to consist of least ten operons carrying almost 40 genes, which may encode for structural and effector proteins (237). These genes are highly conserved among *Chlamydiaceae*, indicative of their importance in the lifestyle of this pathogen, although gene order sometimes varies from species to species, suggesting rearrangement of these loci since acquisition (519).

The T3SS operons are transcribed by the σ^{66} RNA polymerase, although the regulator(s) involved in activation of these promoters is unknown (94). Most of the putative *Chlamydia* T3SS operons are expressed in the mid-developmental cycle during the RB to EB transition; presumably, the T3SS must be assembled prior to EB escape from the host cell, as the T3SS is hypothesized to be important for host cell entry (94; 237). However, there are some outliers. One of the putative operons (CT863/*scc3/copB2/copD2*) is transcribed as shortly as one hour post-infection (94) A later set of genes is also transcribed (which includes the operons *cdsU/lcrD/copN/scc1/malQ*, *cdsJ*/CT560/*cdsL/cdsR/cdsS/cdsT*, and *ssc2*/CT577/*copB1/copD1*) much closer to the end of the developmental cycle.

Adherence and invasion

Chlamydia species adhere to and invade a wide variety of host cells, including the epithelium of the cornea and genital/respiratory tracts. The *Chlamydia* major outer membrane protein (MOMP), the polymorphic membrane proteins (Pmps), and the outer membrane protein OmcB are thought to facilitate adhesion to these host cells, while the T3SS is critical for host cell invasion (203; 351; 539).

In *C. trachomatis* serovar L2, MOMP attaches to heparin sulfate receptors on host cells, a feature shared with viruses (herpes simplex) and other bacteria (*Neisseria gonorrhoeae*) (351; 517; 559; 592). Glycosylation of MOMP appears to be essential for this adherence (528). Not all serovars of *C. trachomatis* bind heparin sulfate, however; for example, *C. trachomatis* serovar E is unaffected by the presence or absence of the ligand (119). Furthermore, MOMP from different species may bind alternative receptors; *C. pneumoniae* MOMP interacts with the mannose 6-phosphate/insulin-like growth factor 2 receptor (436). MOMP attachment is therefore speculated to play a role in defining tissue tropism between species or even serovars.

Several members of the polymorphic membrane protein (Pmp) family also contribute to *Chlamydia* adhesion (203; 238). *Chlamydia* species encode a wide variety of Pmps; in *C. trachomatis*, nine *pmp* genes have been identified, while in *C. pneumoniae, C. felis, C. pecorum, C. abortus, C. caviae,* and *C. psittaci*, at least 20 exist (203; 211; 581). Classified as autotransporters (type V secretion systems), Pmp family proteins are localized to the outer bacterial membrane via an N-terminal signal sequence and subsequently transported across using the C-terminal autotransporter domain, which is thought to then remain embedded in the bacterial membrane (240; 325). The Pmps are highly immunogenic and extremely variable, not only in amino acid sequence but also

length (325). They are characterized by a relatively conserved C-terminal domain and two conserved motifs in the N-terminal domain, one of which (GGAI) is speculated to have a role in adhesion (203; 211). Purified PmpB/C, D and G of *C. pneumoniae* directly bind human epithelial cells (363). Antibodies generated against *C. pneumoniae* PmpD attenuate cell entry, further indicating that this protein may be important in adhesion (575). The functional consequences for variations between the Pmps are still being addressed, but differences in the abundance and amino acid content of Pmps in different *Chlamydia* species or serovars could contribute to differences in tissue tropism. Gomes *et al.* (2006) demonstrated that sequence polymorphisms for six of the nine Pmps of *C. trachomatis* cluster based on ocular or urogenital serovars (203). Furthermore, recombinant *C. trachomatis* PmpG and PmpD proteins were capable of attenuating the infectivity of *C. trachomatis* but not *C. pneumoniae*; conversely, recombinant *C. pneumoniae* Pmp2, Pmp6, or Pmp21 proteins inhibited *C. pneumoniae*, but not *C. trachomatis* infectivity, suggesting that these adhesins are likely species-specific (44).

An outer membrane protein, OmcB, is considered the third adhesion of *Chlamydia* (167; 514; 539). The OmcB proteins carry between one and two heparin-binding motifs at their N-terminal domain, which interact with heparin/heparin sulfate-like glycosaminoglycans (GAGs) on the surface of epithelial cells (167). Despite the fact that the OmcB proteins of *Chlamydia* share roughly 95% identity overall, the N-terminal domain is slightly more variable, with 33-96% identity (238). The variance present in the functional binding domain may be indicative of specificity for different GAG ligands, and could represent another example of tissue tropism. Unlike OmcB from *C. trachomatis* serovar L2, OmcB from *C. trachomatis* serovar E cannot bind heparin

sulfate-type GAGs at all; compared to serovar L2, the OmcB from serovar E carries a single amino acid change within the N domain responsible for this binding inhibition (167). Heparin sulfate-type GAG ligands are present on the deeper layers of squamous epithelium and the basolateral surface of columnar cells lining the genital tract (234). Moelleken and Hegemann (2008) speculate that L2 is able to bind heparin sulfate-type GAGs of these cells to disseminate through the deeper submucosa, eventually reaching the lymph nodes, while serovar E, lacking GAG-binding properties, remains restricted to the superficial genital epithelium (362). However, *C. trachomatis* serovar E OmcB still attaches to the apical side of human epithelial cells, suggesting that OmcB from *C. trachomatis* serovar E utilizes a non-heparin sulfate-type GAG ligand for binding (238).

Host cell entry proceeds following adherence. Nans et al. (2014) examined the pre-entry events of *Chlamydia* using whole-cell cryo-electron tomography (383). Each EB actively organizes into two distinct poles: on one pole, 14-20 T3SS complexes protrude from the bacterial surface, while on the other, narrower pole, non-T3SS complexes also protrude from the bacterial composition, although these unknown structures have not yet been identified. Presumably following T3SS needle contact with the host cell, *Chlamydia* induces host actin rearrangement through the secretion of the tyrosine actin-recruiting protein (Tarp) into the host cell, preceding uptake of the EB (87; 106). The remaining steps of *Chlamydia* entry into the host cell, however, are largely unknown (263). Furthermore, the mechanism of *Chlamydia* entry remains uncertain due to conflicting evidence within the field, and there may actually be more than one mechanism utilized by the bacteria to increase invasion efficiency. Clathrin-mediated endocytosis, caveola-mediated entry, directed phagocytosis, T3SS-mediated entry, and

generalized pinocytosis have all been proposed as possible models (83; 106; 247; 263; 391; 454).

Post-entry T3SS contributions

Very little is known about the contribution of *Chlamydia* T3SS effectors to critical post-entry events, such as development of the inclusion, the EB to RB transition, manipulation of host vesicular trafficking, immune system evasion, the RB to EB transition, and eventual bacterial escape. Only a handful of *Chlamydia* effectors have been experimentally characterized in surrogate T3SSs, such as *Shigella* and *Yersinia*. These proteins include IncA, IncB, IncC, CT847, CopN, and CT694 (102; 175; 255; 520).

Chlamydia IncA, IncB and IncC are secreted by the *Shigella* T3SS (520). Following secretion in *Chlamydia*, the C-terminal hydrophobic domains of these proteins presumably allow for insertion into the maturing inclusion membrane. IncA inhibits soluble *N*-ethylmaleimide-sensitive factor-attachment proteins (SNAREs), a family of host proteins required for membrane fusion (417). Two SNARE-like motifs of IncA are essential for this activity (127). A yeast two-hybrid system identified the host protein Snapin as a possible binding partner for IncB, which suggests that this *Chlamydia* effector may also have a direct role in intracellular trafficking (69; 327). Snapin is targeted to late endocytic compartments where it associates with the SNARE complex and deletion of the *snapin* gene increases the amount of late endocytic lysosome-associated membrane protein 1 (LAMP-1) within the cell. As LAMP and SNARE proteins are required for the fusion of lysosomes with phagosomes, IncA and IncB may

play important roles in preventing phagosome-endosome fusion in the host to protect the development of the mature inclusion (69; 261).

CT847 is expressed during mid-developmental cycle, and can be secreted out of a surrogate *Yersinia* T3SS (102). In a yeast two-hybrid system, and during chlamydial invasion, CT847 associates with Grap2 cyclin D-interacting protein (GCIP), a host protein that is speculated to be important for cell cycle regulation (102; 594). Shortly following infection, GCIP is targeted for degradation, suggesting that CT847 may directly or indirectly deplete the host cell of GCIP, and therefore accelerate the host cell cycle through the G_1/S checkpoint.

CopN, another *Chlamydia* effector, shares homology with *Yersinia* YopN, but the respective phenotypes of these two homologs appear to differ (175; 260). In *Yersinia*, YopN controls T3SS secretion by forming a complex with SycN/YscB/TyeA to effectively block secretion; following host cell contact, YopN is secreted into the host cell (172). Expression of CopN in yeast and mammalian cells disrupts the microtubule network, leading to a G2/M cell cycle arrest. This phenotype was not present following YopN expression, suggesting that the *Chlamydia* CopN may have a unique role in the host cell among these T3SS homologs.

Lastly, the *C. trachomatis* protein CT694 is secreted into the host cell where it interacts with the neuroblast differentiation-associated protein (AHNAK), likely via its C-terminal domain (255). AHNAK proteins bind to G- and F-actin within the eukaryotic cell, and may assist in host cell differentiation and signal transduction (248). Consistent with these phenotypes, expression of CT694 in HeLa cells induces cytoskeletal

alterations, indicating that perhaps this *Chlamydia* effector interferes with AHNAK signaling (255).

DRUG RESISTANCE

Spontaneous mutation events leading to antibiotic resistance are relatively infrequent, and cannot account for the swift spread of resistance through a bacterial community. Horizontal gene transfer plays a much larger role in the rapid dispersion of antimicrobial resistance genes in Gram-negative bacteria such as *Salmonella* and *Shigella* (10; 329). Drug resistance in *Chlamydia*, however, is rare. The exact reason for this difference is unclear, but is possibly due to a lower frequency of genetic exchange with other genera, a trait common among obligate intracellular organisms (393; 453; 475). The obligate intracellular nature of the pathogen means that it is surrounded by multiple membranes, which pose a challenging barrier for DNA to cross. Additionally, *Chlamydia* lacks known conjugation or competence genes, although recombination among *Chlamydia* strains or even species appears to be surprisingly common (277; 278; 360; 475). Recombination events are probably the result of mixed infections *in vivo*, and may help to provide enough additional variation in the population to avoid a potentially deleterious bottleneck.

Although horizontal gene transfer of antimicrobial resistance determinants in *Chlamydia* is rare compared to many other Gram-negative bacteria, the tetracycline resistance phenotype of numerous strains of *C. suis* is a notable exception (315). These isolates carry the *tet*(C) gene, which encodes for an antimicrobial efflux pump (138). Because of the high homology of this gene and the surrounding region to a resistance plasmid from the fish pathogen, *Aeromonas salmonicida*, *tet*(C) may have been acquired

via lateral gene transfer, although how such a rare event occurred is not yet understood. Spontaneous antimicrobial resistant mutants of *Chlamydia* can be isolated *in vitro*, and these mutations can subsequently spread between species (56; 474; 521). In a study by Binet *et al.* (2010), however, spontaneous azithromycin-resistant mutants were not only outcompeted during co-infection with the wild type parent strain, but the mutants alone conferred significantly less inflammation in a guinea pig model of infection, suggesting that the fitness costs associated with certain resistance mutations may be too high for the bacterium to live with (56). With its small genome size, and narrow, specific niche, *Chlamydia* may simply be less flexible regarding alterations to existing genes, and antibiotic resistance may be less feasible in *Chlamydia* compared to organisms with larger, more versatile genomes.

CONCLUDING REMARKS

The *Chlamydia* species are remarkable, versatile pathogens that have evolved to exploit a very specific niche, the eukaryotic host cell. Classical virulence factors such as a T3SS likely support *Chlamydia*'s obligate intracellular lifestyle and concomitantly contribute to disease. Other virulence factors, such as the *tox* cytotoxin, are active in some, but not all *Chlamydia*.

The wide range of hosts and tissues infected by different *Chlamydia* species and serovars suggests that gene presence or variation may play a role in defining the host and tissue tropism of this organism. Several of these factors have been identified, and their roles in pathogenicity will continue to be investigated. Another protein that potentially contributes to *Chlamydia* tissue tropism, the arginine decarboxylase enzyme, will be discussed in the following chapter.

Unfortunately, our understanding of the basic biology of *Chlamydia* remains limited due to barriers in effective genetic manipulation techniques. As genetic systems for this organism continue to improve, significant progress should be made to advance the field.

CHAPTER 8: CHARACTERIZATION OF THE ACTIVITY AND EXPRESSION OF ARGININE DECARBOXYLASE IN HUMAN AND ANIMAL *CHLAMYDIA* PATHOGENS (60)

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Note: The figures, table, and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

Chlamydia pneumoniae encodes a functional arginine decarboxylase (ArgDC), AaxB, that activates upon self-cleavage and converts L-arginine to agmatine. In contrast, most *Chlamydia trachomatis* serovars carry a missense or nonsense mutation in *aaxB* abrogating activity. The G115R missense mutation was not predicted to impact AaxB functionality, making it unclear if AaxB variations in other *Chlamydia* species also result in enzyme inactivation. To address the impact of gene polymorphism on functionality, we investigated the activity and production of the *Chlamydia* AaxB variants. Since ArgDC plays a critical role in the *Escherichia coli* acid stress response, we studied the ability of these *Chlamydia* variants to complement an *E. coli* ArgDC mutant in an acid shock assay. Active AaxB was detected in four additional species: *C. caviae, C. pecorum, C. psittaci*, and *C. muridarum*. Of the *C. trachomatis* serovars, only E appears to encode active enzyme. To determine when functional enzyme is present during the chlamydial developmental cycle, we utilized an anti-AaxB antibody to detect both uncleaved and cleaved enzyme throughout infection. Uncleaved enzyme production peaked around 20 hours post-infection, with optimal cleavage around 44 hours. While the role ArgDC plays in *Chlamydia* survival or virulence is unclear, our data suggest a niche-specific function.

INTRODUCTION

Infection with *Chlamydia*, a genus of Gram-negative obligate intracellular bacteria, may result in ocular, genital, or pneumonic disease, depending on route of entry and bacterial species/serovar. While the majority of *Chlamydia* species are zoonotic, infecting a wide range of mammalian and avian hosts, the *Chlamydia trachomatis* serovars are human-specific pathogens (88; 457). All species undergo a unique biphasic

developmental cycle transitioning between the extracellular, infectious elementary body (EB) and the intracellular, replicative form known as the reticulate body (RB) (2).

Arginine decarboxylases (ArgDCs), which catalyze the conversion of arginine into agmatine, are conserved in bacteria and play dual roles in acid resistance and the metabolism of polyamines such as putrescine (320; 530). In bacteria such as *Yersinia*, functional ArgDC is required to produce biofilms, making this enzyme essential for virulence (416). Two ArgDCs are encoded by *E. coli*: the acid-inducible *adiA* and a constitutive *speA* that functions in polyamine biosynthesis (515). In *Chlamydia*, the only known ArgDC is encoded by *aaxB,* which resides in an operon between the putative porin *aaxA* and the characterized arginine-agmatine antiporter, *aaxC (197)* (Figure 18). Although AaxB is functionally equivalent to *E. coli* AdiA, the enzyme itself is actually a member of the pyruvoyl-dependent ArgDCs (PvlArgDCs), and shares more similarities with ArgDCs from organisms such as *Methanococcus jannaschii* (207).

The AaxB proteins of *Chlamydia pneumoniae* and *C. trachomatis* serovars D and L2 were previously characterized (196; 197). All sequenced *C. pneumoniae* encode a 25 kDa proenzyme, which requires autocleavage between the conserved Thr^{52} Ser^{53} residues to produce 16 kDa α and 9 kDa β subunits. The cleaved subunits are then free to assemble into the active $(\alpha\beta)_3$ complex.

In contrast, *C. trachomatis* serovars D and L2 have inactivated AaxB through one of two independent mutations (196). The Gly^{115}Arg substitution mutation in serovar D (also present and predicted to inactivate AaxB from B/D/G and F) disrupts the necessary auto-cleavage event; in serovar L2, a nonsense mutation midway through the gene results in early truncation. The Gly^{115}Arg mutation present in strains of D was not predicted to

result in enzyme inactivation based on sequence analysis alone, making it unclear if AaxB sequence variations seen in other *Chlamydia* alter AaxB activity.

To further our understanding of this enzyme and determine if inactivation of AaxB is restricted to the human-specific *C. trachomatis* serovars, we completed an activity panel using variant *Chlamydia* AaxB proteins in a surrogate *E. coli* acid shock assay. A pan-chlamydial anti-AaxB antibody was used to detect enzyme production and processing during the developmental cycle using a cell culture infection model. Collectively, our data indicate that non-*C. trachomatis* species (and a single *C. trachomatis* serovar: E) produce active AaxB.

METHODS AND MATERIALS

Strains

Chlamydia strains used in this study include: *Chlamydia muridarum* strain Nigg, *Chlamydia trachomatis* serovar D strain UW-3/CX, *Chlamydia psittaci* strain 6BC, *Chlamydia caviae* strain SP6 (56), and *Chlamydia trachomatis* serovar E strain UW-5/CX. *C. pecorum* strain E58 DNA was provided by Patrik Bavoil (University of Maryland). The previously unreported *aaxB* sequences for *C. caviae* SP6 and *C. trachomatis* E strain UW-5/CX were deposited in Genbank under accession numbers JX287368 and JX287367, respectively. *E. coli* strain MG1655 was used for the acid resistance complementation assays, while *E. coli* Rosetta-gami2 (DE3) (Novagen) was used for AaxB expression and purification.

Cloning of *aaxB*

A pBAD/HisA vector (modified during cloning to remove the histidine tag coding region) (Invitrogen) carrying *aaxB* from *C. pneumoniae* strain Kajaani 6 or *adiA* from *E. coli*

strain MG1655 was provided by David Graham (Oak Ridge National Laboratory). Primers used to amplify the different *aaxB* variants are listed in Table 7. PCR-amplified products were digested and ligated into the NcoI and HindIII sites on the pBAD/HisA vector (again, without the histidine tag). Constructs were then electroporated into Δ*adiA E. coli* strain MG1655. The *aaxB* gene from *C. caviae* also was PCR-amplified (primers listed in Table 7) for cloning into a pET-19b expression vector (Invitrogen). PCR-amplified products were digested and ligated into the NdeI and BamHI sites on pET-19b, then electroporated into *E. coli* strain Rosetta-gami2 (DE3). All constructs were sequence verified at the Biomedical Instrumentation Center at the Uniformed Services University.

***Deletion of* E. coli adiA**

The *adiA* gene was deleted from *E. coli* strain MG1655 using the lambda red method of linear recombination with the primers listed in Table 7 (117). After PCR verification of the constructed Δ*adiA*::kan mutation, the allele was moved into a clean *E. coli* MG1655 background via P1L4 transduction (358). Transductants were selected on LB agar containing 100 μg mL^{-1} kanamycin, verified by PCR, and checked for their acid resistance phenotype.

Acid resistance assay

The acid resistance assay of Castanie-Cornet *et al.* (96), as modified by David Graham (197), was utilized. Strains were grown for 22 hours at 37°C in 5 mL of Brain Heart Infusion (BHI) broth (EMD Millipore) with 0.2% L-arabinose, shaking at 250 rpm in a water bath. To assess the acid resistance phenotype, ~3 x 10^7 bacteria were exposed to acid shock buffer (73 m KH_2PO_4, 17 mM Na_2HPO_4, 10 mM sodium citrate, and 0.8 mM $MgSO_4$, supplemented with 0.4% glucose and 1.5 mM arginine, adjusted to pH 2.5 with

Table 7. List of primers used in this study.

Primer target (cloning vector)	***Sequence (5'-3')***
aaxB of *C. caviae* (pBAD/HisA)	GCATCCATGGCTTACGGAACACGCTAC (Forward) GCGAAGCTTTTACTTAATTGTTGCTG (Reverse)
aaxB of *C. muridarum* (pBAD/HisA)	GCATCCATGGCTTACGGAACTCGTTAT (Forward) GCGAAGCTTTTATTGGATAACAACTG (Reverse)
aaxB of *C. trachomatis* serovar E (pBAD/HisA)	GCATCCATGGCTTACGGAACTCGTTAC (Forward) GCGAAGCTTTTATTGGATAACAGCAG (Reverse)
aaxB of *C. trachomatis* serovar D (pBAD/HisA)	GCATCCATGGCTTACGGAACTCGTTAT (Forward) GCGAAGCTTTTATTGGATAACAGCAG (Reverse)
aaxB of *C. pecorum* (pBAD/HisA)	GCATCCATGGCGTACGGCACACGTTAC (Forward) GCGAAGCTTCTATTGCACGGTTACTG (Reverse)
aaxB of *C. psittaci* (pBAD/HisA)	GCATCCATGGCTTACGGAACACGCTAC (Forward) GCGAAGCTTGCTCCTACTCCTGCTGATG (Reverse)
aaxB of *C. caviae* (pET-19b)	GATCCATATGCCTTACGGAACACGC (Forward) GGTTGGATCCTTACTTAATTGTTGCT (Reverse)
Construction of knockout strain	**Sequence (5'-3')**
Δ*adiA E. coli*	TACTTGCCCGCAACGAAGATTCCTTCATAACCG GGTAAGTGTGTAGGCTGGAGCTGCTTC (Forward) GGCGTAATGTTATTTAAACAATTACGCCTTCAG CGGAATCATATTGAATATCCTCCTTAGTTCC (Reverse)

HCl) and then diluted and titered in acid dilution buffer (acid shock buffer without glucose or arginine, adjusted to pH 7.0) either directly after exposure to the acid shock or at 1 hour post-acid shock. Bacterial dilutions were plated on LB agar with antibiotics as required and incubated overnight at 37°C. Acid resistance was expressed as the percentage of viable bacteria remaining after one hour acid shock compared to the number of viable bacteria determined immediately following acid shock.

Production of AaxB antibody

The highly conserved chlamydial AaxB peptide ^{137}HAKMWLKKSLQHELDLRS^{154} (part of the α subunit) was commercially synthesized by Pierce Custom Antibody production service and used to raise polyclonal rabbit antibodies using the standard 90-day protocol.

Purification of *C. caviae* AaxB

E. coli Rosetta-gami2 (DE3) was transformed with either pET-19b carrying *aaxB* from *C. caviae* or empty vector and grown in LB containing 100 μg mL^{-1} ampicillin to an OD_{600} of 0.6, then set on ice for 10 minutes. AaxB expression was induced with 1 mM IPTG for 23 hours at 20°C, then bacteria were collected by centrifugation for 10 minutes at 10K rpm at 4°C. Bacteria were resuspended in 10 mL equilibration buffer (50 mM monobasic sodium phosphate, 300 mM sodium chloride, and 10 mM imidazole, pH adjusted to 7.4) with 1x protease inhibitor (Roche) and 1x phosphatase inhibitors 2 and 3 (Sigma). Bacteria were lysed via sonication, centrifuged for 10 minutes at 10K rpm at 4°C, and the supernatant passed through a 0.45 μm filter (Millipore). Two mL of HisPur^{TM} cobalt resin (Thermo Scientific) was applied to the supernatant and the batch method of purification was carried out as per manufacturer's instructions. Purified protein samples were eluted in a total of 4 mL elution buffer (50 mM sodium phosphate, 300 mM sodium chloride,

500 mM imidazole, pH adjusted to 7.4), then applied to a 3K Amicon filter (Millipore) for concentration. Samples were resuspended in 50 mM Bis-Tris buffer (pH 6.0), and purified protein samples were quantified by the Bio-Rad Protein Assay (Bio-Rad). Protein identity and purity was assessed using SDS-PAGE followed by Coomassie Brilliant Blue Staining or Western blotting with the anti-AaxB antibody (at a 1:250 dilution).

Detection of chlamydial AaxB in EBs and during infection

Chlamydia were grown in and harvested from mouse fibroblast L2 cells. EBs were titered using an infection forming unit assay (IFU) and stored at -80°C in Sucrose Phosphate Glutamic acid buffer (SPG) (7.5% w/v sucrose, 17 mM Na_2HPO_4, 3 mM NaH_2PO_4, 5 mM L-glutamic acid, pH 7.4) until use (56). For time course experiments, L2 cells were infected at an MOI of 5 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected (196). Samples were disrupted directly in Laemmli buffer and run on 12% SDS-PAGE gels for Western blot analysis with either anti-AaxB antibodies or anti-Hsp60 antibodies (provided by Dan Rockey, Oregon State University) (602). To detect AaxB in EBs, bacteria were disrupted in Laemmli buffer and 1 x 10^7 IFU were used per SDS-PAGE gel lane.

RESULTS

Alignment of *Chlamydia* AaxB

The AaxB sequences from the available *Chlamydia* genome projects were aligned to assess amino acid variability. All *Chlamydia* species have at least one sequenced strain available in the Genbank database except *C. suis*, a porcine pathogen. As many strains

within the same species or serovar had identical protein sequences, duplicates were discarded and only unique AaxB sequences are shown in Figure 18.

Despite differences in amino acid composition, all AaxB variants carried the highly conserved $Thr^{52}Ser^{53}$ cleavage site. *C. trachomatis* serovars A/B/D/F and G carry a missense mutation, a glycine to arginine substitution ($Gly^{115}Arg$) that was shown to abrogate cleavage of the protein and therefore activity in the serovar D variant (196). In *C. trachomatis* serovar L2, an ochre codon at position 128 truncates the gene in mid-open reading frame. This truncated protein lacks activity (196). Neither *C. trachomatis* serovar E, nor any of the remaining *Chlamydia* species, carry either of the known mutations that have been shown to inactivate AaxB. However, there are variations in the amino acid sequence of these proteins compared to the amino acid sequence of the active *C. pneumoniae* AaxB. As the missense mutation in *C. trachomatis* serovars A/B/D/F and G was not indicative of protein inactivation, we measured the activity of the remaining variants.

Activity of *Chlamydia* AaxB

Previously, Giles and Graham demonstrated that expression of functional AaxB from *C. pneumoniae* can rescue an *E. coli* Δ*adiA* mutant from acid shock, demonstrating activity of the *Chlamydia* enzyme in a surrogate system (197).To test the remaining *Chlamydia* variants, an Δ*adiA* knockout of *E. coli* MG1655 was constructed and transformed with wild type *E. coli adiA* or *Chlamydia aaxB* genes cloned into a vector under control of an arabinose-inducible promoter. The different AaxB variants from *C. caviae, C. muridarum, C. trachomatis* serovar E, *C. psittaci*, and *C. pecorum* were tested in the acid resistance assay, with AaxB variants from *C. pneumoniae* and *C. trachomatis*

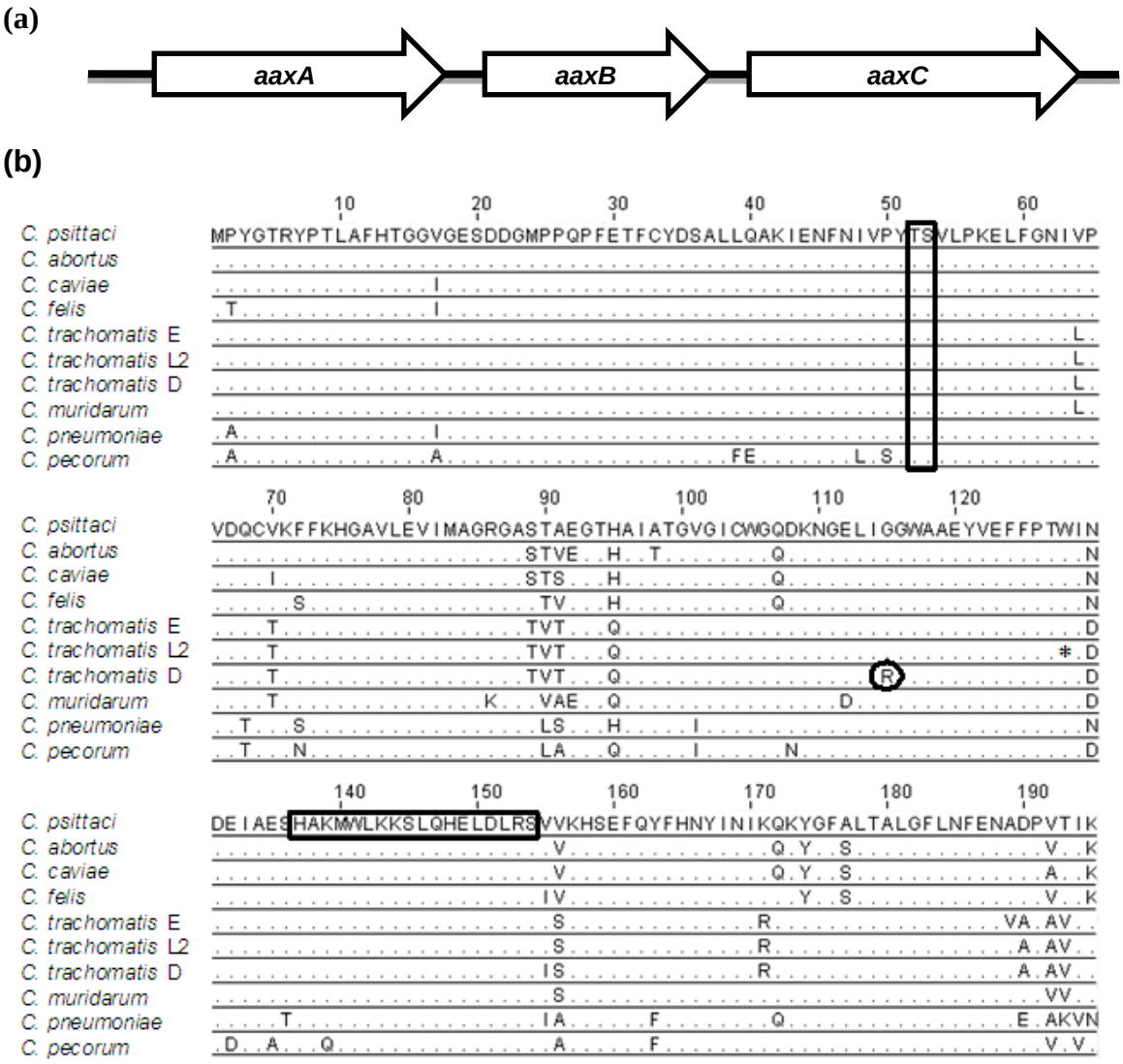


Figure 18. Gene organization and protein alignment.

(a) *aaxB* is transcribed in an operon with *aaxA*, an outer membrane protein of unknown function, and *aaxC*, a characterized arginine-agmatine antiporter (Smith and Graham, 2008). (b) ClustalW alignment of different unique *Chlamydia* AaxB proteins, representative of all fully annotated strains found on the NCBI server as of June 23, 2012. The conserved Thr[52]Ser[53] autocleavage site essential for activity is boxed. * indicates the nonsense mutation in *C. trachomatis* serovar L2 that results in early truncation of the protein. A circle indicates the glycine to arginine mutation that abrogates cleavage and therefore activity; this mutation is conserved in all *C. trachomatis* A, B, D, and G lineages. Rabbit polyclonal antibody was raised against the highly conserved 18 amino acid peptide (outlined in the box) present in all AaxB sequences except the truncated *C. trachomatis* serovar L2.

serovar D serving as positive and negative controls, respectively (Figure 19). All *Chlamydia* AaxB tested restored acid shock survival in the *E. coli* Δ*adiA* mutant, suggesting that *C. caviae, C. muridarum, C. trachomatis* serovar E, *C. psittaci,* and *C. pecorum* all encode active enzyme.

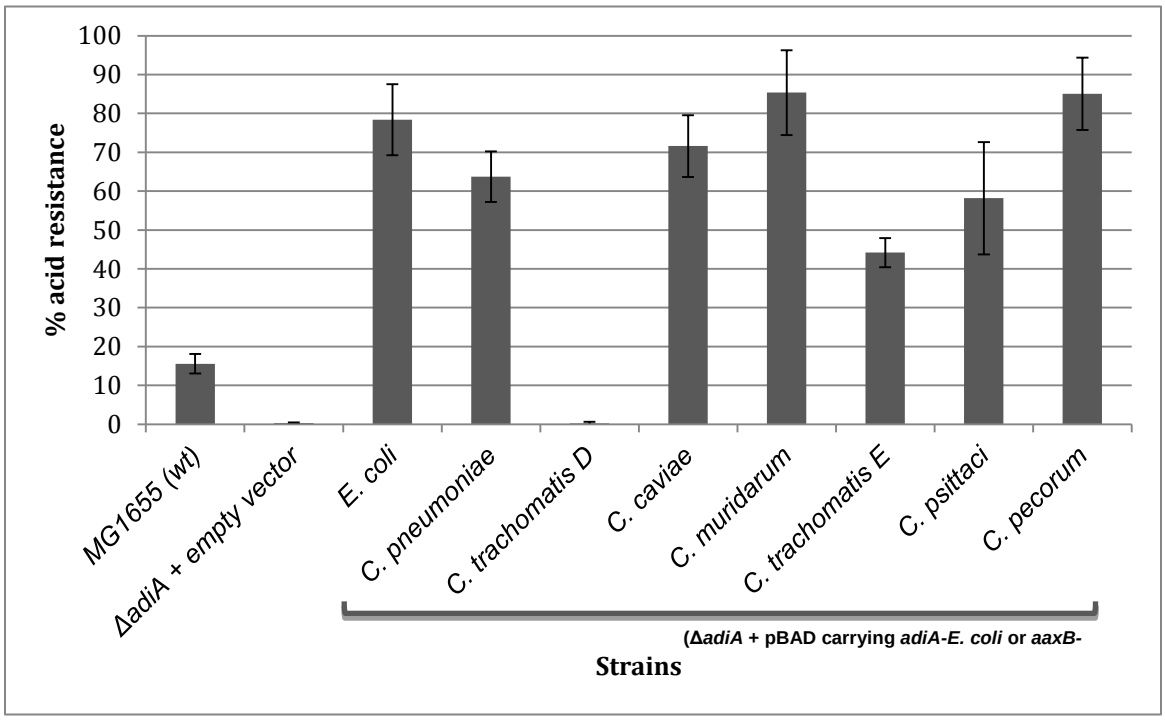
Protein expression and cleavage of the AaxB variants was measured via Western blotting with anti-AaxB antibody (Figure 19). All constructs used in the acid shock experiments expressed uncleaved AaxB protein, and each active AaxB variant was capable of auto-cleavage as evidenced by detection of the α fragment (Figure 19); i.e. the cleavage profile correlates with acid resistance.

Time course expression of AaxB in *Chlamydia*

The timing of AaxB protein production and cleavage, and therefore activity, during infection is unknown. To determine when active enzyme is present during the chlamydial developmental cycle, the highly *Chlamydia*-conserved peptide ^{137}HAKMWLKKSLQHELDLRS^{154} was used to produce rabbit polyclonal antibodies. This antibody recognizes both the inactive, uncleaved proenzyme form of AaxB, as well as the activated α subunit, and therefore cleavage of this protein can be directly measured during infection.

L2 cells were infected with *C. caviae*, and the expression and cleavage of AaxB into active subunits over the course of infection was studied (Figure 20). A unique band of ~20 kDa representing uncleaved proenzyme was initially detected at 20 hours post-infection, with very little cleaved protein (<20 kDa) appearing. Over the next 24 hours, this ratio slowly shifted, and by 44 hours post-infection, the majority of protein was in the cleaved, active state.

(a)


% acid resistance
100
90
80
70
60
50
40
30
20
10
0
MG1655 (wt)
ΔadiA + empty vector
E. coli
C. pneumoniae
C. trachomatis D
C. caviae
C. muridarum
C. trachomatis E
C. psittaci
C. pecorum
(ΔadiA + pBAD carrying adiA-E. coli or aaxB-
Strains

(b)

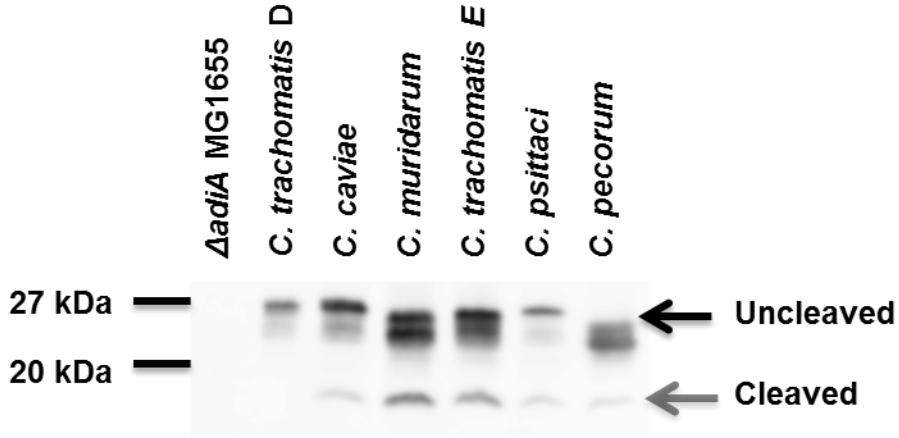

ΔadiA MG1655
C. trachomatis D
C. caviae
C. muridarum
C. trachomatis E
C. psittaci
C. pecorum
27 kDa
20 kDa
Uncleaved
Cleaved

Figure 19. Activity analysis of AaxB proteins.

(a) Strains were grown for 22 hours at 37°C in the presence of 0.2% arabinose. ~3 x 10^7 cfu was exposed to acid shock buffer containing 0.4% arginine (pH 2.5) for one hour, and percent survival determined by viable plate counts. Strains were tested in three independent experiments performed in triplicate. Wild type MG1655 was significantly acid resistant compared to the Δ*adiA E. coli* empty vector control using a standard t-test with unequal variance ($p<0.005$); all other strains, with the exception of *C. trachomatis* serovar D, were able to complement the Δ*adiA E. coli* mutant over wild type. The difference in acid resistance between the Δ*adiA E. coli* strain expressing AaxB from *C. trachomatis* serovar D and the empty vector control was not statistically significant ($p>0.1$). (b) AaxB protein production and cleavage was measured using Western blotting with the anti-AaxB antibody. Molecular weight markers are shown to the left of the blot, and the proenzyme and α fragments (if present) are indicated with black and gray arrows, respectively.

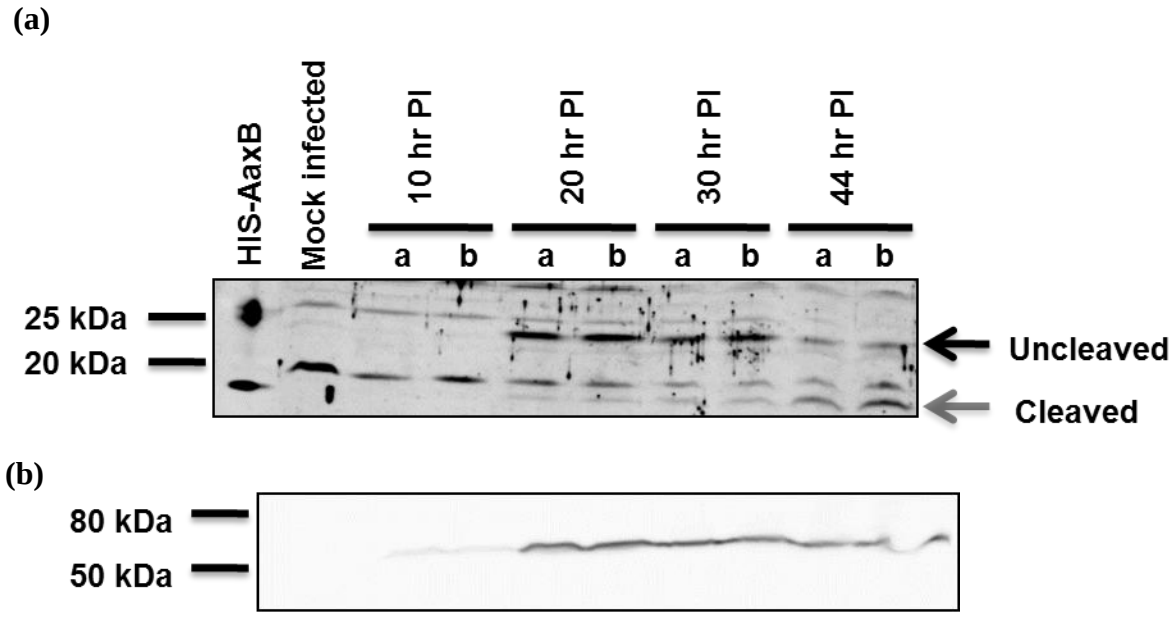


Figure 20. Time course of *C. caviae* AaxB production.
L2 cells were infected at an MOI of 5 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected. Western blotting was performed with either anti-AaxB antibodies (a) or anti-Hsp60 antibodies (b). Purified his-tagged AaxB is shown in the first lane. Two parallel-run infection samples are shown for each time point. Proenzyme and the α fragment are indicated by black and gray arrows to the right of the blot. Protein molecular weight marker positions are shown to the left of each blot.

Interestingly, this pattern did not necessarily hold true across all the *Chlamydia* species (Figure 21). In *C. muridarum*, while the majority of uncleaved protein also appeared at 20 hours post-infection, cleaved protein production likewise peaked at this time, then waned at subsequent time points. *C. psittaci* produced very little detectable cleaved protein.

Cleavage of AaxB also was assessed in EBs in comparison to samples from cells infected for 20 hours when full-length protein appears to be the predominant species (Figure 22). The cleaved form predominates in EBs, and very little, if any, detectable proenzyme remains. Despite equal loading of bacteria, AaxB was undetectable in *C. trachomatis* serovar D.

DISCUSSION

Previously, a functional arginine decarboxylase enzyme, AaxB, was identified and characterized in *C. pneumoniae* (197). In this study, we demonstrate that several additional *Chlamydia* species, including *C. caviae, C. muridarum, C. psittaci,* and *C. pecorum*, encode functional AaxB. Although previous publications established that the majority of the *C. trachomatis* serovars encode nonfunctional AaxB due to one of two inactivating mutations (196), we now show that the AaxB variant of *C. trachomatis* serovar E is capable of cleavage and activity.

AaxB undergoes maximal autocleavage during the mid to late *Chlamydia* developmental cycle, with slight variations on timing between the different species. At the extremes, optimal cleavage of *C. muridarum* AaxB occurs around 20 hours post-infection, with *C. caviae* AaxB cleaving around 44 hours. Although cellular conditions for autocleavage are not yet clear, timing of cleavage may be influenced by differences

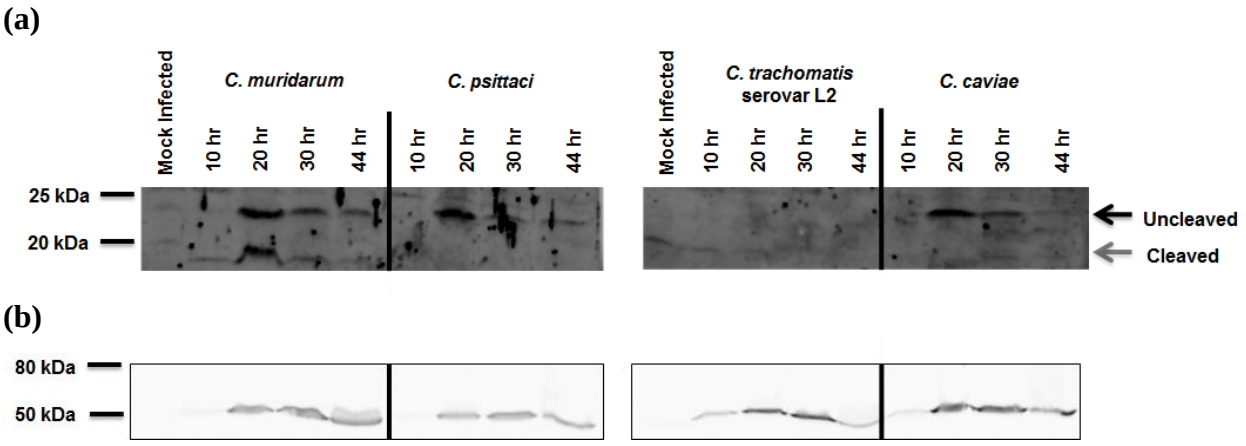


Figure 21. Time course of AaxB production by different *Chlamydia* spp.
L2 cells were infected at an MOI of 10 (10 hr samples), an MOI of 1 (20, 30, and 44 hr samples), or mock infected. At the indicated times, samples were disrupted directly in Laemmli buffer and run on 12% SDS-PAGE gels. Protein was detected by Western blotting with either anti-AaxB antibodies (a) or anti-Hsp60 antibodies (b). Full length AaxB is indicated by the black arrows and processed AaxB is indicated by the grey arrows. Protein molecular weight marker positions are shown to the left of each blot.

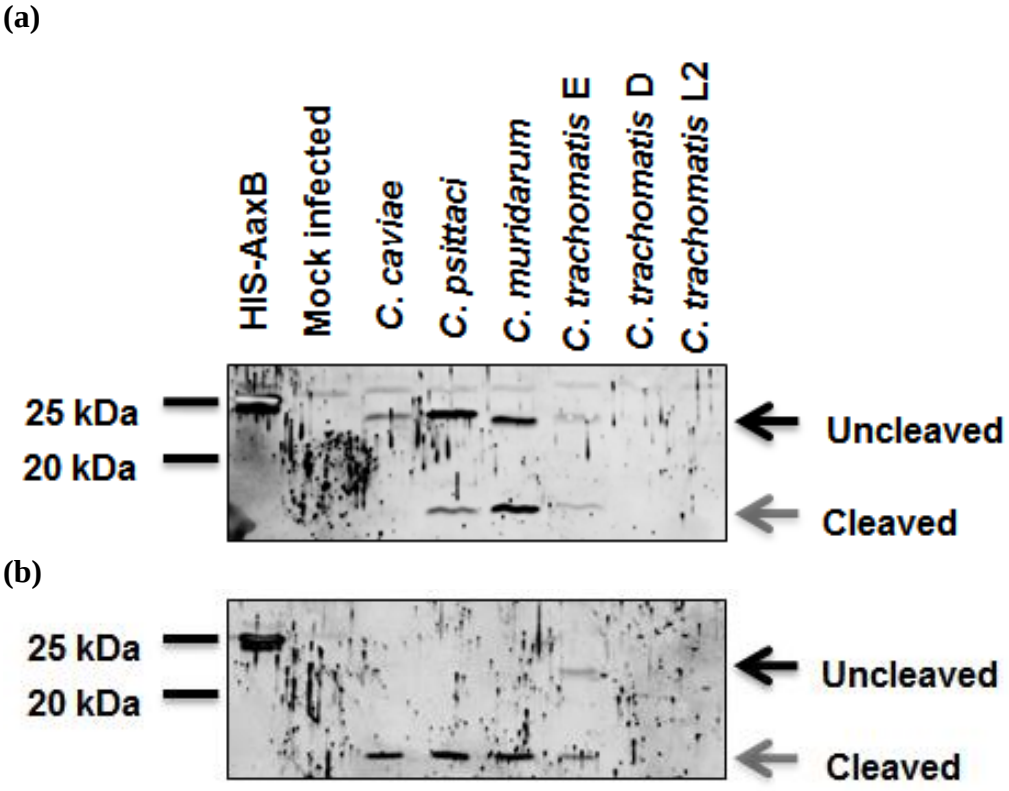


Figure 22. AaxB detection in EBs and 20-hour post infection samples.
L2 cells were infected at an MOI of 1 or mock infected for 20 hrs (a). $1x10^7$ purified EBs were loaded per lane in (b). Samples were disrupted directly in Laemmli buffer prior to 12% SDS-PAGE/Western blotting analysis with anti-AaxB antibodies. Proenzyme and the α fragment are indicated by black and gray arrows to the right of the blot. Protein molecular weight marker positions are shown to the left of each blot.

in amino acid composition between variants or post-translational modification.

We were unable to detect AaxB from *C. trachomatis* serovar D. Since this enzyme appears to be nonfunctional, production of AaxB would squander bacterial energy resources. Due to the early truncation of the *C. trachomatis* serovar L2 AaxB, the anti-AaxB antibody, which was developed against a conserved peptide after the truncation, would not recognize this serovar if truncated protein is produced. However, previous data using an *E. coli* surrogate expression system indicates that this protein may not be produced (196).

The total protein level of AaxB in *C. trachomatis* serovar E also appeared to be lower than the non-*C. trachomatis* variants (possibly indicating decreased expression levels), and the acid resistance phenotype of the serovar E AaxB producing strain was the weakest of the complementing strains. As the only *C. trachomatis* serovar expressing active AaxB, it is possible that the serovar E strains represent an intermediate phenotype between isolates that have maintained or lost enzyme functionality. Several studies suggest that there is no association between infections with *C. trachomatis* serovar E and presence or absence of clinical infection or specific symptoms, although this serovar is one of the most prevalent worldwide (82; 370; 556). As the other genital serovars (D, F-K) occupy the same niche, it is unlikely that serovar E requires active AaxB when the other serovars have lost functionality. This, coupled with the low AaxB levels detected during *in vitro* infection, suggest that although *C. trachomatis* serovar E currently retains active AaxB, this serovar may be in the process of inactivating this enzyme.

While *C. pneumoniae* and many of the non-*C. trachomatis* serovars retain an active ArgDC, the function of this enzyme in *Chlamydia* remains obscure. Although

ArgDCs in other bacteria play roles in acid resistance and/or polyamine metabolism, neither function appears relevant to *Chlamydia*. The *Chlamydia* inclusion remains at neutral pH throughout infection, so encounters with acidic environments are unlikely (18; 210; 486). Additionally, there are no known *Chlamydia* enzymes able to metabolize agmatine, such as the agmatine ureohydrolase, and therefore AaxB cannot contribute to polyamine synthesis. Finally, in certain cell lines, addition of exogenous agmatine alone may provide protection against cellular apoptosis (28), but investigation in our laboratory suggests that this is likely not a factor during *Chlamydia* infection (data not shown).

As Giles and colleagues have speculated previously, the most likely function for the arginine decarboxylase system during *Chlamydia* infection is depletion of host cell arginine reserves (197). L-arginine, a substrate for the nitric oxide synthase (iNOS) in macrophages and granulocytes, catalyzes the production of the reactive nitrogen intermediate nitric oxide (NO), which may play a role in decreasing bacterial infectivity and/or inhibiting the dissemination of *Chlamydia* to systemic sites (264; 345; 595). Removal of the substrate L-arginine (which would be degraded to agmatine and pumped back into the cytosol in counter-exchange for arginine by AaxC) could therefore promote *Chlamydia* survival and/or fitness, particularly in strains that are known to infect and replicate within these specialized host cells, such as *C. pneumoniae* and *C. psittaci* (451; 593). The timing of cleavage, and presumably corresponding activity, of AaxB in these strains may correlate with optimal iNOS activation in infected macrophages, and ultimately allow *Chlamydia* to avoid the detrimental consequences of NO production prior to bacterial exit from the host cell. Alternatively, the presence of processed AaxB in

EBs may indicate that EBs are ‘pre-loaded’ with functional AaxB that is used to protect against NO production during the immediate-early stage of infection.

ACKNOWLEDGEMENTS

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CHAPTER 9: EXTENDED DISCUSSION AND FUTURE DIRECTIONS

The majority of discussion points for each of the three projects presented (*S*mip construction and characterization, the search for novel AVGs in *Shigella*, and the *Chlamydia* arginine decarboxylase characterization) lie in their respective chapters above. This final section will provide extended commentary regarding the *S*mip characterization and arginine decarboxylase projects. Potential future directions for all three projects will also be discussed.

SHIGELLA PROJECTS

*S*mip characterization

There are several *S*mip phenotypes that must be addressed before this tool is used extensively, as they may present limitations to its usefulness. *S*mip stability is the primary concern, as the *S*mip was considerably less stable than the large virulence plasmid. After 24 hours growth in TSB at 37°C, roughly 60% of BS889 colonies were no longer resistant to kanamycin and chloramphenicol, indicative of plasmid loss (data not shown). Wild type *S. flexneri* 2a, in contrast, did not demonstrate significant loss and/or inactivation of the large virulence plasmid until 72 hours, consistant with previously published data (488). Furthermore, while loss of the large virulence plasmid was reliant on the temperature-dependent expression of the T3SS, loss of the *S*mip was not, as loss was significantly demonstrated during serial passage at 30°C when the T3SS genes are no longer expressed. During longer experiments, this problem can be partially alleviated by growing *S*mip-carrying strains in the presence of antibiotics required to select for the plasmid (i.e., kanamycin and/or chloramphenicol); similarly, plaque size increases when chloramphenicol is added to the agarose overlay of *S*mip-carrying strains during the plaque assay (data not shown). This instability may be due to the absence of a stability

gene(s) encoded on pINV outside of the *S*mip. The *mvpA/mvpT* genes encode an effective toxin-antitoxin post-segregational killing system on the pINV (481). Although the *mvpA/mvpT* genes cannot explain the *S*mip instability phenotype, as the toxin is not encoded on the *S*mip, the existence of a post-segregational killing system on the pINV suggests that perhaps *Shigella* has developed ways of maintaining this large plasmid. Previous work suggests that, when the T3SS is expressed, there is a significant fitness cost to maintain the plasmid in the absence of selective pressure, and the presence of stability systems might prevent pINV from being lost too quickly (488). pINV does encode two separate plasmid partitioning systems (*parAB* and *stbAB*), although little research has been done to study the functionality or importance of these systems in stable plasmid maintenance (79). It is possible that the single plasmid partitioning system encoded on the pCC1Fos backbone may not be sufficient for stable plasmid maintenance of the *S*mip. Additionally, other as-yet-uncharacterized ORFs encoded on pINV, yet absent from the *S*mip, could also contribute to plasmid stability.

*S*mip-carrying *Shigella* bacteria also demonstrate a temperature-independent stress response increase compared to wild-type *Shigella*, as indicated by increased filamentation frequency; this filamentation phenotype was also present in the *E. coli*/*S*mip strains (data not shown). At this time, it is unknown if the stress response and stability issues stemming from the presence of this plasmid are linked.

The *S*mip will continue to be utilized for effector characterization in our laboratory as the need arises. It would be additionally useful to determine which gene(s) contribute to the full invasive phenotype, as we did see slight but significant invasion attenuation with BS875 compared to the wild type 2457T. *virA* and *ipgB2*, both absent

from the *S*mip, would be good potential candidates to study. The activation of host calpain proteases by VirA triggers host cytoskeleton remodeling, which could play a role in early invasion events; IpgB2, meanwhile, works in concert with IpgB1 (present on the *S*mip) to activate small GTPases during infection, also resulting in cytoskeletal rearrangements (52; 146; 295). Although single-deletion mutants of *virA* or *ipgB2* do not confer any significant invasion defects, functional redundancy of effectors that target the actin cytoskeleton during infection may be masking their respective contributions (149; 219). A combined Δ*ipgB1*/Δ*ipgB2* double mutant is significantly attenuated for plaque assay formation, even though Δ*ipgB1* or Δ*ipgB2* single mutants demonstrate negligible defects, indicating that at least IpgB1 and IpgB2 play redundant roles in this process (219). Therefore, although single *virA* or *ipgB2* deletion mutants did not demonstrate any invasion defects, the absence of both *virA* and *ipgB2* from the Smip may explain the invasion defect seen in BS875 (Figure 7).

AVG search in commensal *E. coli*

As we were unable to develop adequate screens to identify novel AVGs in the commensal *E. coli* isolates, we decided to pursue the identification of AVGs in enteroinvasive *E. coli* (EIEC) strains. As described in chapters three and four, the EIEC strains represent an intermediate phenotype between the commensal *E. coli* and the pathogenic *Shigella* (308). Preliminary data acquired from the EIEC strains M4163 and 4608-58 (gifts from Susan Leonard and Keith Lampel at the Food and Drug Administration) demonstrate that these strains invade at roughly 13.8% and 7.8% respectively, of wild type *S. flexneri* 2a strain 2457T (data not shown) (316). As expected this attenuation was not as severe as the invasion inhibition of the *E. coli*/*S*mip strains

(Figures 12 and 16). Furthermore, we were able to assess intercellular spread of these strains. Plaque diameters of M4163 and 4608-58 were approximately 41.0% and 38.6%, respectively, of the wild type *S. flexneri* 2457T strain (data not shown). Taken together, these data indicate that cell entry and intercellular spread in the EIEC strains are impaired compared to *S. flexneri* 2457T. A transposon mutant library will be constructed in EIEC 4608-58, and mutants screened to attempt to identify genes which, when interrupted, restore plaque diameter to the level of wild type *S. flexneri* 2457T.

Additionally, in collaboration with Dr. Keith Lampel's laboratory at the Food and Drug Administration, we are undertaking a more comprehensive bioinformatics analysis to identify pseudogenes across the *Shigella* species to assemble a more complete list of candidate AVGs. Current pseudogene identification in *Shigella* is largely reliant on annotation methods, which often incorrectly annotate pseudogenes as functional (374). Several bioinformatics comparisons between *Shigella* and *E. coli* strains have been published, but these comparisons are either extremely limited in the number of strains analyzed or compare *Shigella* to lab-adapted K-12 strains such as MG1655 (170; 576) In our analyses, we will be comparing 209 *Shigella* and 14 EIEC genomes, in addition to several commensal *E. coli* strains. Genes present in EIEC and the commensal *E. coli* strains, but absent in the majority of *Shigella* isolates, will be identified. Furthermore, as IS element insertions and gene truncations are responsible for the majority of gene inactivation events in *Shigella*, a separate analysis between *Shigella*, EIEC, and commensal *E. coli* strains will compare ORF length between orthologous genes (317; 576; 603). Inactivation by IS elements may lengthen or shorten ORF length, while nonsense mutations result in truncated (shorter) ORFs; consequently, ORF length can be

used to identify potential pseudogenes. Therefore, these two analyses should help to identify the majority of *Shigella* pseudogenes either entirely lost or inactivated due to IS element insertion or truncation. Once a list of candidate AVGs is assembled, functional candidate AVGs will be cloned and expressed in wild type *Shigella*, and the resulting effect on *in vitro* virulence phenotypes will be assessed.

CHLAMYDIA PROJECT

Arginine decarboxylase characterization

At the time of the arginine decarboxylase paper publication, a complete genome sequence of *C. suis* was unavailable to investigate the gene sequence of *aaxB*. Recently, a fully sequenced genome was published (133). Alignment of the *C. suis* AaxB protein with the other *Chlamydia* AaxBs revealed that it contained neither the nonsense mutation of *C. trachomatis* serovar L2, nor the missense mutation that prevents cleavage of AaxB in the *C. trachomatis* A, B, D, and G serovars (BLAST analysis, 03/23/15, http://blast.ncbi.nlm.nih.gov/Blast.cgi) (196). In *C. suis*, the full-length *aaxB* gene appears to be intact, although the resulting AaxB protein (192 amino acids) does not share 100% identity with any other *Chlamydia* AaxB. The *C. suis* AaxB appears to be most closely related to the AaxB from *C. muridarum* (94% identity), which is unsurprising given the proposed close evolutionary history of these two species (133). However, only one amino acid change is entirely unique to *C. suis*; at position 122, all known *Chlamydia aaxB*s encode for a valine, while *C. suis aaxB* encodes for an isoleucine. Although the functionality of this enzyme would need to be experimentally determined, valine and isoleucine are both in the aliphatic class and share similar biochemical properties, making this amino acid substitution conservative and therefore

likely not inactivating. We hypothesize that the arginine decarboxylase enzyme in *C. suis* is probably functional, which would parallel our hypothesis that AaxB remains conserved in the zoonotic *Chlamydia* pathogens and is slowly undergoing inactivation in the human-specific species.

It is intriguing that *C. trachomatis* serovar E maintains a functional AaxB, although the other *C. trachomatis* serovars appear to have inactivated this protein via missense or nonsense mutations (196). However, this may be an example of evolution in progress, in which some strains have inactivated the gene, and others have not (yet). We hypothesize that loss of active AaxB in the *C. trachomatis* serovars is due to lack of positive selection, and the corresponding inactivation events were likely due to neutral rather than negative selection, as there is no evidence currently to suggest otherwise. In this instance, loss of functional AaxB would not pose a significant advantage over the parent wild type strain, and eventually the coding sequence for this gene would decay. If there is no selective advantage to maintain a functional AaxB in any of the *C. trachomatis* serovars, we postulate that eventually *aaxB* will be inactivated in *C. trachomatis* serovar E.

Future experiments for this project include determination of the function AaxB plays during the infection process of the zoonotic *Chlamydia* pathogens. Giles *et al.* (2007) suggested that *C. pneumoniae* avoid nitric oxide exposure in granulocytes and macrophages by utilizing a functional AaxB to degrade the substrate arginine required for nitric oxide synthesis (196). To explore this hypothesis, *Chlamydia* survival in host macrophages would need to be investigated. Ideally, we would inactivate *aaxB* in *C. pneumoniae*, then infect macrophages with wild type *C. pneumoniae* or the Δ*aaxB*

isogenic mutant. Bacterial survival curves would be measured, and the corresponding levels of nitric oxide (NO) in the infected macrophages could be assessed using a commercial colorimetric kit (Sigma). Until very recently, the *Chlamydia* field has lacked the genetic tools to construct targeted chromosomal deletions or inactivations. In 2013, Johnson and Fisher published a novel method to insertionally inactivate *incA* in *Chlamydia*, which could be utilized to insertionally inactivate *aaxB* in *C. pneumoniae* for these experiments (274). If we are unable to inactivate *aaxB* in *C. pneumoniae*, we could alternatively utilize wild type *C. trachomatis* serovar D and an isogenic strain expressing functional *aaxB* on a plasmid. If AaxB functions to deplete host arginine stores, bacteria expressing active AaxB should be able to reduce NO levels and avoid macrophage killing.

APPENDIX

ANTIVIRULENCE GENES: INSIGHTS INTO PATHOGEN EVOLUTION THROUGH GENE LOSS (62)

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K. A. Bliven and A. T. Maurelli both contributed ideas for the general outline of the chapter. K. A. Bliven reviewed the literature and wrote the chapter.

Note: The figures, table, and reference numbers have been adjusted to follow the format of this dissertation.

ABSTRACT

The emergence of new pathogens and the exploitation of novel pathogenic niches by bacteria typically require the horizontal transfer of virulence factors and subsequent adaptation – a 'fine-tuning' process –for the successful incorporation of these factors into the microbe's genome. Function of newly-acquired virulence factors may be hindered by expression of genes already present in the bacterium. Occasionally, certain genes must be inactivated or deleted for full expression of the pathogen phenotype to occur. These genes are known as antivirulence genes (AVGs). Originally identified in *Shigella*, AVGs have improved our understanding of pathogen evolution and provided a novel approach for drug and vaccine development. In this review, we revisit the AVG definition and update the list of known AVGs, which now includes genes from pathogens such as *Salmonella*, *Yersinia pestis*, and the virulent *Francisella tularensis* subspecies. AVGs encompass a wide variety of different roles within the microbe, including genes involved in metabolism, biofilm synthesis, lipopolysaccharide modification, and host vasoconstriction. More recently, the use of one of these AVGs (*lpxL*) as a potential vaccine candidate highlights the practical application of studying AVG inactivation in microbial pathogens.

INTRODUCTION

All species evolve over time. The evolution or acquisition of new genes enhances an organism's ability to adapt within novel niches and ultimately augments the organism's fitness. In the case of microbial species, exposure to new environments, competition with other species for limited resources, and the need to evade predators and/or host immune defenses all contribute to selective pressures that determine which

organism – and, more importantly, which set of genes - will endure. The rise of antimicrobial resistance is a classic example of pathoadaptation. The horizontal transfer of antibiotic genes on plasmids or pathogenicity islands provides many modern-day pathogens an edge in survival within the clinical setting.

Gene loss can be just as critical to microbial survival as gene acquisition, although less attention has been given to this facet of the evolutionary process. Most commonly, loss-of-function gene mutations result from bacterial adaptation to a more specific niche. As certain gene products or pathways become superfluous in this new environment, neutral mutations are allowed to accumulate in unnecessary genes with negligible consequence on bacterial fitness. In the earliest stage of this reductive evolution, organisms start to accumulate pseudogenes in unnecessary pathways, although they still retain the majority of genes necessary for a free-living bacterium. Next, at an intermediate stage in reductive evolution, all or many of the superfluous genes become inactivated, but the nonfunctional remnants of these genes still persist. At this stage, expression of such pseudogenes may already be in a state of erosion. The intermediate stage of reductive evolution can be observed in specific niche-adapted organisms known to boast a high frequency of pseudogenes, such as *Shigella flexneri*, *Salmonella enterica*, and *Yersinia pestis* (129; 414; 576). Over time, regions no longer encoding functional genes may be gradually eliminated from a bacterial genome (367). At the final stage of reductive evolution, bacteria possess considerably smaller genomes than their predecessors and few pseudogenes, indicating that they are reaching the end of this evolutionary pathway (317). Such final stage organisms include endosymbionts and obligate intracellular pathogens, such as *Buchnera*, *Mycobacterium leprae* and

Chlamydia trachomatis, which have adapted to scavenge key nutrients from their hosts rather than directly synthesize substrates, and have accordingly lost numerous biosynthetic pathways (108; 334; 497; 513).

A second evolutionary force driving gene loss also occurs in microbial pathogens. As virulence is a critical factor for the continued survival of these organisms, the expression of any gene that interferes with virulence will be detrimental to the pathogen's fitness. If the selective pressure against such a gene is significant, the function of that gene must be silenced for virulence, and ultimately bacterial fitness, to persevere. This concept is known as **antivirulence** (339). An **antivirulence gene (AVG)** is a gene whose expression in a pathogen is incompatible with the virulence of that pathogen (Box 1). Therefore, an AVG must be inactivated, deleted, or differentially regulated to prevent its expression from interfering with the pathogen's virulence.

In this review, we highlight the AVGs that have been described in the time since the last comprehensive review (339) and present a list of known antivirulence genes (Table 8). These include not only AVGs that were lost as a result of commensal-to-pathogen evolution, such as in the case of *Escherichia coli* to *Shigella*, but also during pathogen-to-pathogen evolution, as in the case of *Yersinia pseudotuberculosis* to *Yersinia pestis*. While we limit our discussion to AVG discoveries in bacterial species, antivirulence has the potential to occur in other pathogens such as parasites, although the genetic complexities of these organisms make AVGs more difficult to identify.

WHAT IS (AND ISN'T) AN ANTIVIRULENCE GENE

Before reviewing the most recent additions to the AVG list, we must first correct some common errors made in the classification of such genes (Box 1). First, suppressors

Box 1. Definition of an antivirulence gene.

Box 1 – Definition of an antivirulence gene

What is an antivirulence gene?

An antivirulence gene is a gene whose expression in a pathogen is incompatible with the virulence of that pathogen. In the pathogen, an antivirulence gene is inactivated, deleted, or differentially regulated so it cannot interfere with the virulence of that organism.

What is not an antivirulence gene?

Suppressors: **Certain genes, when inactivated under experimental conditions or determined to be nonfunctional in a limited number of natural isolates, can lead to an increase in virulence, a phenomenon commonly known as hypervirulence.**

Genes not functional in the ancestral species: **Antivirulence genes must have been present in the ancestral species before being inactivated or lost. As the ancestral strain/species may not always be available to study, the nearest related extant species may be acceptable for comparison. For example, *Shigella* species arose from an ancestral *E. coli* strain and *Y. pestis* diverged from *Y. pseudotuberculosis*. Pseudogene formation (and consequently antivirulence gene formation) in *Shigella* and *Y. pestis* may be studied by comparing their genomes or phenotypes to the corresponding extant species (*E. coli* and *Y. pseudotuberculosis*, respectively).**

Genes that have undergone genetic decay due to close association with the known antivirulence gene(s): **Only that gene(s) which causes the antivirulence phenotype will be considered the antivirulence gene. Once that gene(s) has been lost, other associated genes may become superfluous in the absence of a fully functional system and may therefore also decay. This process is part of reductive evolution.**

Table 8. Known antivirulence genes (AVGs).

Pathogen:	Gene(s):	Presence of functional gene inhibits:	Reference(s):
Shigella/EIEC	*nadA/nadB*	T3SS secretion	(433; 434)
Shigella/EIEC	*cadA*	PMN transepithelial migration, ShET1/ShET2 enterotoxin activity, phagolysosome escape	(122; 171; 342; 346)
Shigella	*speG*	Oxidative stress survival	(39)
Shigella	*ompT*	Stability of IcsA	(381)
Salmonella	*lacI*	Expression of SPI-2 genes	(157)
Burkholderia pseudomallei	*araA-araH*	Virulence in the Golden Syrian hamster model	(366)
Yersinia pestis	*rcsA; nghA*	Biofilm formation; biofilm stability	(154; 155; 522)
Yersinia pestis	*lpxL*	Protection from the host immune response	(365)
Francisella tularensis/ holarctica	*pepO*	Systemic spread of the pathogen	(223)

are not AVGs. The term 'hypervirulent' is often used to describe a strain that exhibits a significant increase in virulence compared to either the parental wild type strain or related strains within the same species. Such an increase in virulence may be due to the functional loss of one or more genes, and this phenomenon has been documented in wild type strains or discovered through genetic manipulation of strains within the laboratory (34; 258; 447). However, such genes are still functional in either the majority of wild type strains within the same species, or, in the case of lab-induced hypervirulence, in the parent wild type strain, and therefore do not fit our criteria for AVGs. Instead, these genes are best defined as regulators of virulence or virulence suppressors.

Second, AVGs must have originally been both widely present and functional in the ancestral species. *ompT*, previously characterized as an antivirulence gene in *Shigella*, presents an example of the difficulties in applying this criterion (381). OmpT, a surface protease, degrades the bacteria protein IcsA, consequently inhibiting *Shigella* cell to cell spread. *ompT* is situated within the DLP12 cryptic lambdoid-like prophage, just downstream of the gene *envY*. This prophage is carried by most, but not all, lineages of *E. coli* (109/144 strains in the EcoCyc database carry *ompT*; data retrieved on July 11, 2012, EcoCyc version 16.1) (291). None of the sequenced *Shigella* strains carry the DLP12 cryptic prophage at this position, so we cannot say with certainty that all or indeed any of the ancestral *Shigella* strains ever had, and subsequently lost, *ompT*. Although we tentatively label *ompT* as a *Shigella* antivirulence gene, the inclusion of *ompT* in this category remains debatable.

There are other obvious difficulties in applying this second criterion. In certain cases, we simply will not have an extant species available to study. In the case of host-

restricted *Shigella*, which is postulated to have arisen from multiple lineages within free-living commensal *E. coli*, evaluation of nutrient requirements within the host, conserved metabolic pathways, and readily accessible genome sequences make identification of putative AVG targets more straightforward. In the absence of a reference species to compare to the pathogen of interest, it becomes much more challenging to differentiate putative AVGs from those that were lost due to reductive evolution.

Finally, any gene that has undergone genetic decay as the result of close association with the AVG will not be considered an AVG in its own right. *cadA*, for example, is the AVG linked to absence of the lysine decarboxylase system in *Shigella*. The closely associated *cadB* gene, which encodes a lysine-cadaverine antiporter, is also inactivated in nearly all species. However, the presence of *cadB* does not appear to have any effect on virulence alone. With the loss of functional *cadA*, the lysine decarboxylase system in *Shigella* was lost, and in the absence of any selective pressure to maintain a functional *cadB*, mutations were allowed to accumulate in this gene. However, note that in certain pathways or systems, more than one gene may be involved in the antivirulence phenotype. For example, both *nadA* and *nadB* of *Shigella* encode enzymes that catalyze the synthesis of quinolinic acid, a small molecule inhibitor of *Shigella* virulence (434). If either gene is inactivated, the pathway to quinolinic acid synthesis is lost, so both *nadA* and *nadB* are considered AVGs for *Shigella*.

THE REPERTOIRE OF AVGS IN BACTERIAL PATHOGENS

Shigella, Enteroinvasive *Escherichia coli* (EIEC), Enterohemorrhagic *E. coli* (EHEC), and Shiga-toxin producing *E. coli* (STEC)

The genus *Shigella* likely arose between 35,000 to 270,000 years ago from multiple ancestral *Escherichia coli* lineages (438). During its evolution from an

extracellular resident of the mammalian colon to an intracellular pathogen, *Shigella* acquired a large 220 kb virulence plasmid that harbors the genes required for successful invasion, replication, and dissemination inside host cells, in addition to the induction of the host inflammatory response that is critical to the bacteria's life cycle (487). As the majority of *Shigella* virulence factors are plasmid-encoded, and the rest are localized to distinct pathogenicity islands, it has been suggested that *Shigella* evolved from commensal *E. coli* strains. In addition to gene acquisition, *Shigella* accumulated a plethora of pseudogenes. On average, each strain appears to have lost functionality of approximately 200 genes (597). As *E. coli* remains the paradigm species for bacterial research and *Shigella* has so recently evolved, there is a unique opportunity to study both reductive evolution and antivirulence in *Shigella*.

Enteroinvasive *E. coli* (EIEC), one of the five classical pathogenic *E. coli* subtypes, share a particularly close relationship to *Shigella*. EIEC is a non-motile, facultative intracellular pathogen, harbors the same 220 kb virulence plasmid as *Shigella*, and is usually grouped with *Shigella* as a single pathovar within *E. coli* (308). Only a few metabolic differences, including mucate and acetate production, separate these two species. Many of the AVGs of *Shigella*, such as those encoding for lysine decarboxylase activity and quinolinic acid synthesis, have also been mutated or lost from EIEC (92; 433). A previous review focused on the loss of two antivirulence genes in *Shigella* and EIEC: a surface protease, *ompT*, and the lysine decarboxylase enzyme, which is encoded by *cadA* (339). No new publications have addressed *ompT*, so we will not discuss that particular AVG further. Since the last review, several additional antivirulence genes have been identified in *Shigella*, including *nadA/nadB* and *speG* (39; 434) (Figure 23).

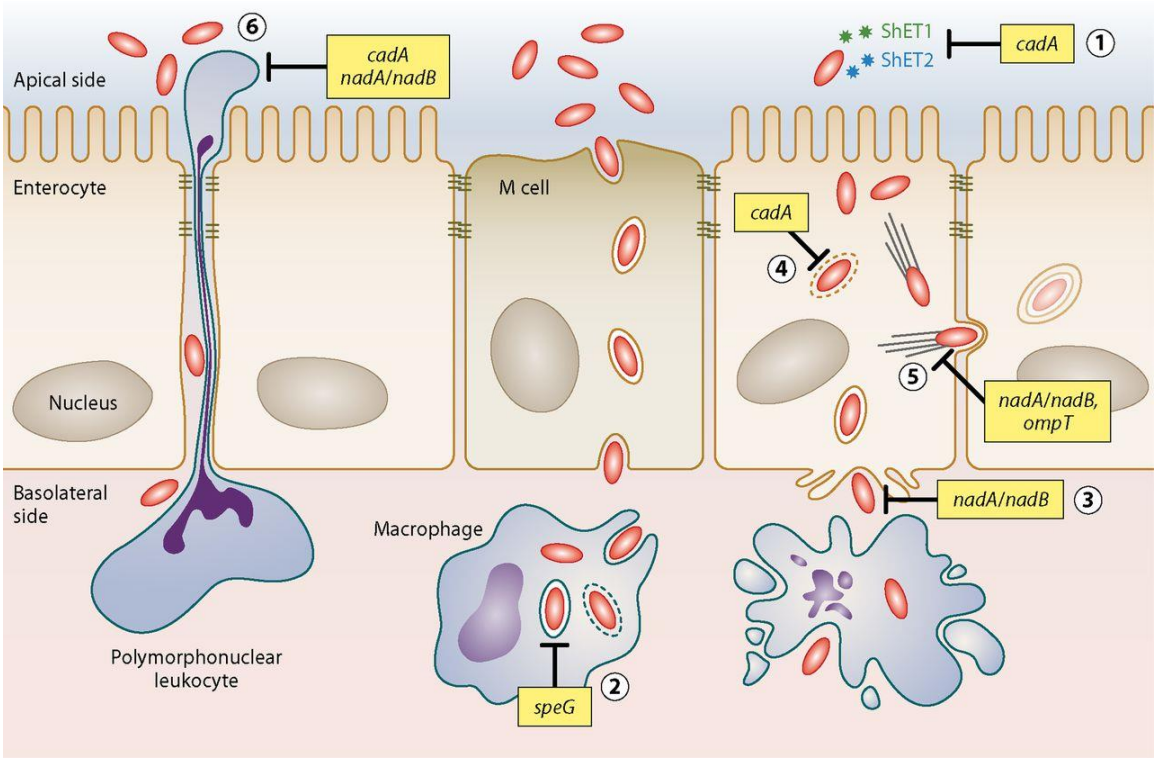

Figure 23. Inhibition of pathogenesis in *Shigella*.

Pathogenesis phenotypes interrupted by *Shigella* AVGs. The product of the lysine decarboxylase reaction, cadaverine, inhibits ShET1/ShET2 enterotoxin activity (part 1), phagosome escape (part 4), and PMN transepithelial migration (part 6). Another small molecule, quinolinic acid, is the product of the *nadA*/*nadB* enzymatic reactions and inhibits both *Shigella* invasion (part 3) and intracellular spread (part 5). Inactivation of *speG*, which encodes the spermidine acetyltransferase, allows spermidine to accumulate within the phagosome and ultimately promotes bacterial survival in the macrophage (part 2). OmpT, an outer membrane protease, cleaves IcsA from the bacterial surface, preventing actin tail polymerization and inhibiting cell-to-cell spread (part 5).

The lysine decarboxylase enzyme converts the amino acid lysine to the polyamine cadaverine. This exchange functions as one of several acid resistance systems in *E. coli*. In contrast, a functional lysine decarboxylase system has been uniformly lost from all *Shigella* species (122) (Figure 24). This system consists of two operons: *cadBA*, encoding for a lysine/cadaverine antiporter and a lysine decarboxylase enzyme, respectively; and *cadC*, the transcriptional activator of *cadBA*. A wide variety of different mutations or deletions have resulted in the loss of *cadA*, and often *cadB* and *cadC*, from all known *Shigella*/EIEC species (122). Correspondingly, absence of lysine decarboxylase activity is one of the classical hallmarks that distinguishes *Shigella*/EIEC from other *E. coli* species in the clinical laboratory.

Expression of a functional *cadA* in wild type *Shigella*, or exposure to physiologically-relevant levels of exogenous end product, cadaverine, results in a disruption of ShET1/ShET2 enterotoxin activity, inhibition of PMN transepithelial migration, and a blockage in bacterial phagosome escape, all of which are critical contributors to *Shigella* virulence inside the host (171; 342; 346). The mechanism(s) of action of cadaverine in regards to inhibition of *Shigella* virulence, while currently under investigation, remains unknown.

Although approximately 90% of *E. coli* species encode a functional lysine decarboxylase system, the *cad* operon has also been inactivated or lost in certain strains of enterohemorrhagic *E. coli* (EHEC) and Shiga toxin-producing *E. coli* (STEC). Reconstitution of a functional lysine decarboxylase system in these strains decreases their ability to adhere to host cells (546; 562). In these studies, the increased adherence seen in *cadA* negative strains appears to be the result of increased expression of the outer

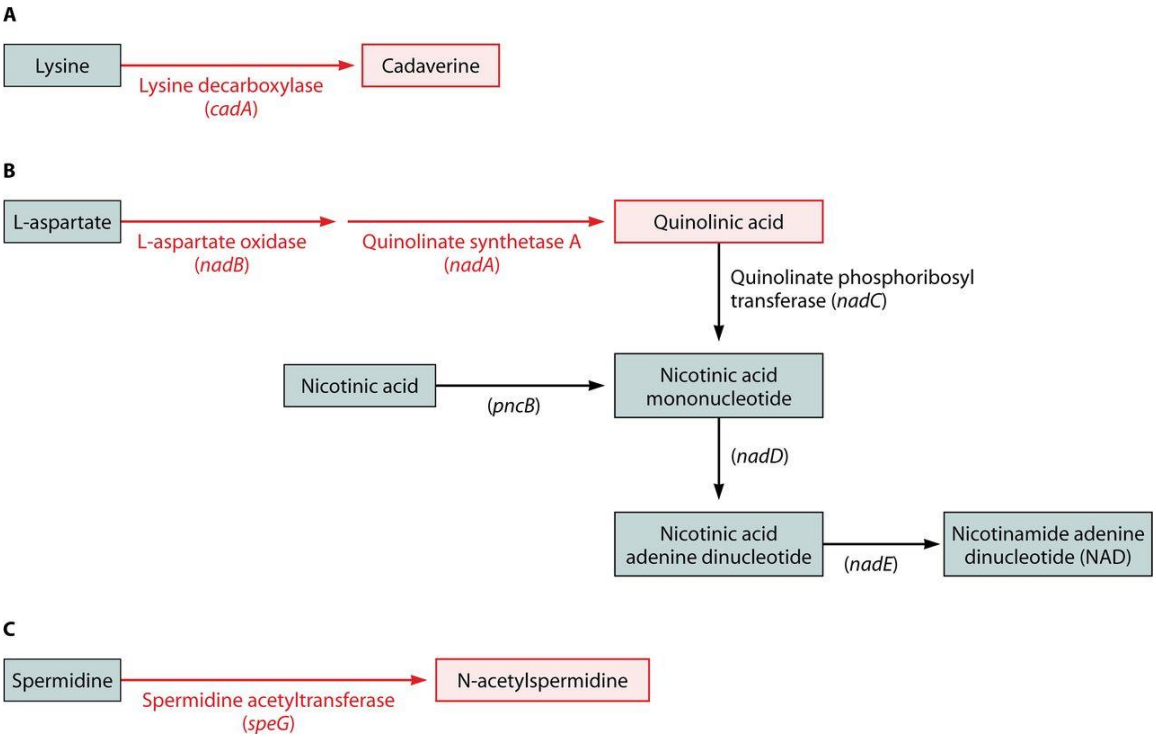

Figure 24. Metabolic pathways lost in *Shigella*.
Compounds or enzymes still present in *Shigella* are marked in black; those that have been lost are marked in red. (A) Lysine decarboxylation. (B) Biosynthetic and salvage NAD pathways. (C) Spermidine metabolism.

membrane adhesin intimin, although the exact mechanism of action is not yet clear. This phenomenon, while not considered antivirulence, would suggest that the lysine decarboxylase system plays an important role not only in acid tolerance in *E. coli*, but also in the regulation of numerous virulence factors of both *Shigella* and *E. coli*. Although *cadA* is currently not inactivated across all EHEC and STEC strains, which precludes the classification of *cadA* as an AVG in these organisms, these events may define a case of evolutionary transition. If selective pressure to lose this locus continues, the *cadA* gene may eventually be considered an AVG in these pathogens.

In order for *E. coli* to synthesize nicotinic acid mononucleotide, a precursor of the essential nicotinamide adenine dinucleotide (NAD), the enzymes L-aspartate oxidase (encoded by *nadB*) and quinolinate synthetase A (encoded by *nadA*) are required. By contrast, *Shigella* has a strict nutritional requirement for nicotinic acid for growth on minimal media, suggesting that *Shigella* relies on a salvage pathway for NAD (194). When the sequences of genes required for *de novo* NAD synthesis in *E. coli* were compared to the *Shigella* genome, it quickly became apparent that all *Shigella* species have inactivated either *nadA, nadB,* or both genes (433; 434). These inactivations take the form of multiple amino acid substitutions, complete or partial deletions of *nadA* or *nadB,* or insertion sequence (IS) elements interrupting either or both genes. The wide variety of different genetic alterations utilized by *Shigella* to inactivate these genes suggests that there was strong selective pressure to lose this part of the NAD pathway. To compensate, *Shigella* bypasses this early block in the pathway by importing exogenous nicotinic acid and converting it to the NAD precursor, nicotinic acid mononucleotide, through the actions of nicotinate phosphoribyltransferase (434) (Figure 24).

Prunier *et al.* showed that one of the intermediates in the *de novo* synthesis pathway, quinolinic acid, attenuates both *Shigella* invasion and intracellular dissemination, in addition to blocking PMN transepithelial migration (434). Secretion of the *Shigella* type three secretion system (T3SS) effectors, such as IpaB and IpaC, is greatly decreased in the presence of quinolinic acid, suggesting that inadequate secretion of *Shigella* effectors most likely contributes to these phenotypes. Interestingly, this inhibition is limited to the *Shigella* T3SS. Other organisms with a T3SS, such as *Salmonella enterica*, enteropathogenic *E. coli* (EPEC) or *Y. enterocolitica* do not exhibit virulence inhibition in the presence of quinolinic acid. The exact mechanism of quinolinic acid inhibition upon *Shigella* virulence is not yet known.

Finally, the ability to acetylate another polyamine, spermidine, has also been lost in the evolution of *E. coli* to *Shigella* (39). Barbagallo and colleagues utilized a particularly novel approach in an attempt to locate new *Shigella* AVG. Working on the hypothesis that the transcriptional activators of the major virulence plasmid, such as VirF, might also incidentally activate other genes on the *E. coli* chromosome, they conducted a global transcriptional analysis to identify VirF-activated *E. coli* genes, and then determined whether or not those genes were correspondingly inactivated in *Shigella*. Genes that were turned on in *E. coli* by VirF, but were functionally lost in *Shigella*, were identified as putative AVG. One of the genes identified via this method was *speG*, which encodes a spermidine acetyltransferase that transfers an acetyl group onto the polyamine spermidine to produce N-acetylspermidine (Figure 24).

speG is inactivated in all *Shigella* lineages through a startling number of missense mutations, IS insertions, and complete deletions of *speG* (39). Unlike loss of the lysine

decarboxylase system, loss of *speG* was not designed to prevent synthesis of an inhibitory end product. Instead, *Shigella* likely inactivated or lost this enzyme because the pathogen actually requires increased amounts of the substrate, spermidine, for intracellular survival. Therefore, blocking any pathway that metabolizes this polyamine would be favored by the emerging pathogen.

In wild type strains from each of the four *Shigella* species (*S. flexneri, S. boydii, S. sonnei*, and *S. dysenteriae*), absence of functional SpeG prevents spermidine metabolism, and correspondingly, spermidine levels are significantly higher in wild type *Shigella* than in wild type *E. coli*. When the active *speG* in *E. coli* is replaced with the inactive *speG* from *S. dysenteriae*, spermidine levels rise to levels seen in the wild type *Shigella* strains (39). Increased spermidine levels correlate with increased survival in response to oxidative stress such as exposure to hydrogen peroxide in macrophages, which is particularly critical to this microbe's pathogenesis. In the early stages of infection, *Shigella* is taken up by macrophages, where it survives and eventually escapes the unfavorable conditions of the phagosome, which include reactive oxygen species such as superoxide and hydrogen peroxide. Ultimately, the pathogen induces pyroptosis of the host macrophage to invade neighboring epithelial cells (524).

In a mouse macrophage model, wild type *Shigella* is able to out-compete the same *Shigella* strain expressing the active *speG* from *E. coli* (39). Taken together, these data suggest that the accumulation of spermidine may be critical for *Shigella* intracellular survival *in vivo*, and loss of a functional *speG* was crucial for *Shigella* pathoadaptation to its new host niche. Although the mechanism for this survival is not yet clear, Barbagallo and colleagues determined that in the presence of increased spermidine, the expression of

katG also rose. The hydroperoxidase encoded by *katG* is critical for antioxidant defense in bacteria. Its expression is mediated by the OxyR stress response, which is induced in the presence of polyamines such as spermidine. Thus, spermidine-mediated induction of OxyR may account for the increased macrophage survival seen in wild type *Shigella.* Taken together, these data support the identity of *speG* as an AVG in *Shigella*.

Salmonella

E. coli and *Salmonella* diverged from a common ancestor approximately 100 million years ago (134). The genus *Salmonella* comprises two main species: *Salmonella enterica*, which includes the serovars Typhi, Typhimurium, and Enteritidis; and *Salmonella bongori*, a closely-related species that infects cold-blooded animals. It has been postulated that these two species diverged approximately 25-40 million years ago, and that subsequent development of *S. enterica* ultimately required the acquisition of new virulence factors by lateral gene transfer (43). Among these, *S. enterica* obtained SPI-2, a chromosomally-located pathogenicity island that contains the genes necessary for intracellular survival and replication.

One of the characteristics that distinguish *E. coli* and *Salmonella* is the ability of *E. coli* to ferment lactose, while *Salmonella* is traditionally thought of as a non-fementer. Paradoxically, both organisms thrive in the human gut where dietary lactose is readily available as an energy source. It is unknown if the ancestral lineage that gave rise both to *E. coli* and *Salmonella* was a lactose-fermenter, or if this ability was obtained only by *E. coli* after the divergence of these two organisms.

In *E. coli*, the *lac* system contains four genes, three of which are located in an operon: *lacZ* (β-galactosidase), *lacY* (lactose permease), and *lacA* (transacetylase). The

fourth gene, *lacI*, encodes the *lac* operon repressor, and negatively regulates the system under lactose-deplete conditions.

Although the majority of *Salmonella* strains are unable to ferment lactose, there have been isolated reports of strains that can (348). Upon closer inspection of the lactose-fermenting *Salmonella*, it was determined that the functional *lacZYA* in these particular strains are likely carried on transmissible plasmids rather than in the chromosome (163). Furthermore, the vast majority of these strains lack *lacI*. The only known *S. enterica* strain (ST-2) that synthesizes a functional LacI has decreased repression efficiency (163). *S. bongori* also appears to be undergoing loss of the *lac* system: of the four genes, only *lacI* and *lacZ* are present, and transcriptional analysis suggests that *lacI*, at least, is a pseudogene (157).

Eswarappa and colleagues introduced a fully functional *lacI* into *Salmonella* to study its effect on pathogenicity. When *lacI* is expressed in *S. enterica* lacking a functional *lac* operon, bacterial virulence is significantly reduced in a murine typhoid fever model compared to the wild type parent (157). Moreover, although the bacteria expressing *lacI* are able to invade as well as their wild type parent, they lack the ability to proliferate inside murine macrophages, suggesting that expression of *lacI* interferes with post-invasion events. A microarray analysis uncovered that several genes of the SPI-2 pathogenicity island, which harbors a T3SS thought to be critical in the assembly of the *Salmonella*-containing vacuole, are down-regulated in the presence of functional *lacI*. These down-regulated genes include *ssaK*, a T3SS apparatus protein; *sseB*, a T3SS effector that makes up part of the SPI-2 translocon; and *spiC*, a T3SS effector essential for SPI-2 mediated secretion of SseB, SseC, and SseD (249; 601).

Although the exact mechanism of action has yet to be elucidated, the ability of LacI to bind operator sequences is not required for the AVG phenotype, suggesting LacI does not function as a direct repressor to down-regulate these genes. Instead, it is possible that LacI may inhibit expression of these genes via protein-protein interactions with other transcription factors. The functional consequence of the down-regulation of these T3SS genes is decreased survival for bacteria residing in the macrophage vacuole, and ultimately marks *lacI* as an AVG of *Salmonella*.

Yersinia

Yersinia pestis, the agent of bubonic and pneumonic plague, exploits a flea vector for its mammalian host transmission cycle. In contrast, the closely-related *Y. pseudotuberculosis*, which presents as a much milder enteric pathogen in humans, is transmitted through the fecal-oral route, and (like *Y. pestis*) preferentially infects lymphoid tissue. Despite drastic differences in mode of transmission, these species share at least 97% genetic identity, and an ancestral *Y. pseudotuberculosis* strain is believed to have given rise to *Y. pestis* (6; 100). Intriguingly, the evolution of *Y. pseudotuberculosis* to *Y. pestis* has affected only a handful of genes (243). The acquisition of new genes by *Y. pestis* has been well-documented, and includes the horizontal transfer of two plasmids, pPCP1 and pMT1, which are vital for bacterial dissemination and flea vector survival, respectively (607).

Loss-of-function mutations have also played a significant role in the evolution of *Y. pestis* from *Y. pseudotuberculosis*. Approximately 200 genes are inactivated in *Y. pestis* based on a genome comparison with *Y. pseudotuberculosis* (100). Notably, *Y. pestis* lost genes whose products repress biofilm synthesis (*rcsA*) and enhance biofilm

degradation (*nghA*), underscoring the importance of biofilm stability in this pathogen's lifestyle. Furthermore, *Y. pestis* has also lost *lpxL*, a gene that encodes for an acyltransferase that modifies bacterial lipopolysaccharide (LPS). In the absence of this protein, the bacteria are unable to stimulate host TLR4, which plays a critical role in pathogen evasion of the host immune response (Figure 25).

Y. pestis must generate a specialized *hms*-dependent biofilm over the spines of the proventriculus of the flea in order to optimize the low efficiency of transmission from flea to mammalian host (243). Even in the preferred vector, *Xenopsylla cheopis*, the transmission rate of infected fleas only reaches around 50%, and biofilm formation across the proventriculus is considered a requirement for even this relatively low rate (243). Not only does an extracellular matrix permit the bacteria to aggregate and adhere to midgut epithelial cells, the biofilm also blocks ingested food from entering, ultimately starving the flea. As the flea attempts, time and time again, to unsuccessfully feed and sate its appetite, the frequency of new host bites rises and, correspondingly, the probability of bacterial transmission increases. Not surprisingly, blocked fleas transmit *Y. pestis* at a much higher rate than their unblocked counterparts (244).

Because of the critical role biofilm formation plays in *Y. pestis* transmission, genes that function in biofilm breakdown or negatively regulate biofilm synthesis would conflict with the ability of *Y. pestis* to pass from vector to host. Two biofilm-associated genes – *rcsA* and *nghA* – are inactivated in all known *Y. pestis* strains, and fit the criteria for *Y. pestis* AVGs (154; 522).

rcsA is a negative regulator of biofilm synthesis (522). RcsA, an accessory protein for the Rcs histidine kinase bacterial phosphorelay system, increases the repression

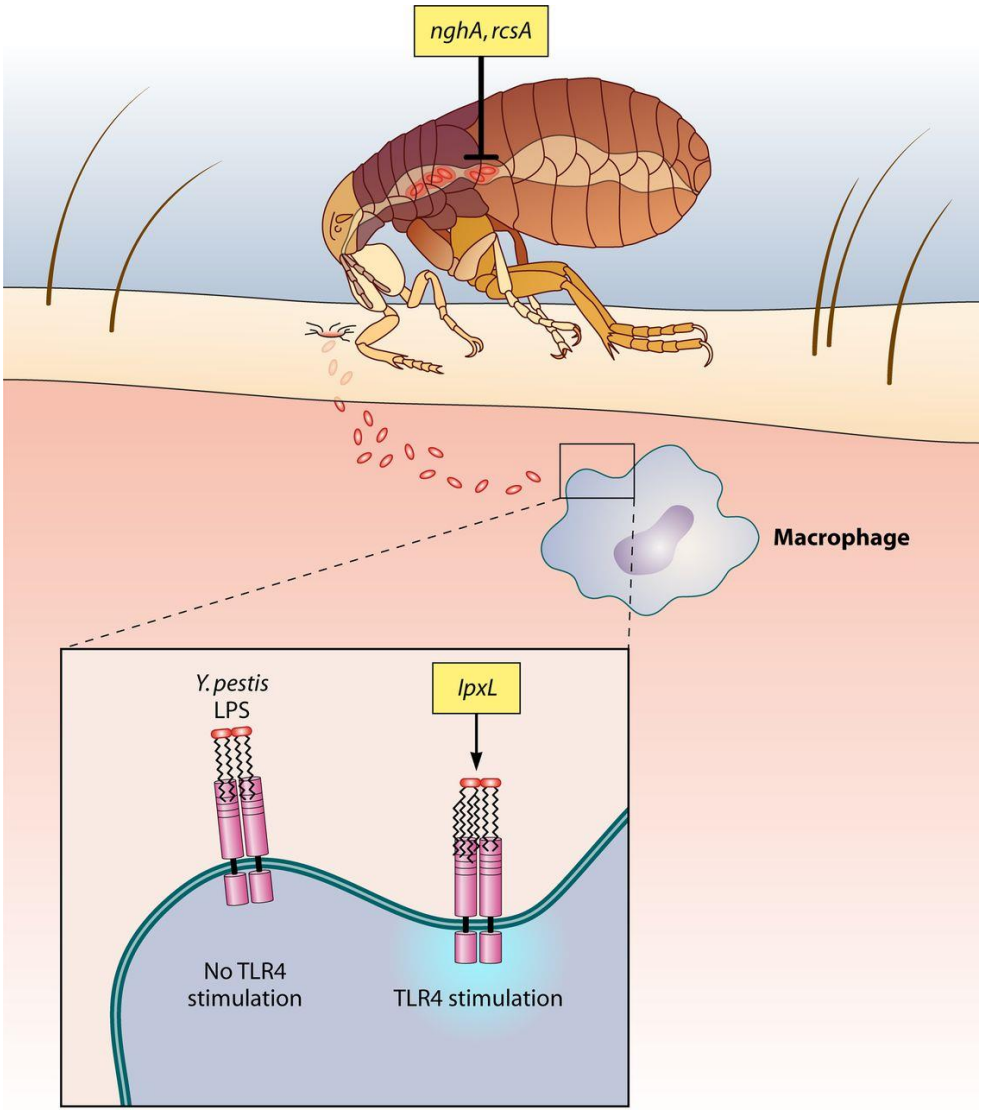

Figure 25. Inhibition of pathogenesis in *Yersinia.*
nghA and *rcsA* encode proteins that inhibit biofilm formation in *Yersinia*: NghA directly degrades formed biofilm, and RcsA increases the repression ability of RcsB, an inhibitor of biofilm formation. Inactivation of these genes allows *Yersinia* to form a biofilm on the proventriculus of the flea, enabling bacterial transmission. LpxL mediates hexa-acetylation of lipid A on bacterial LPS, thus activating TLR4 and stimulating the host immune response to this pathogen. Loss of *lpxL* in all sequenced *Y. pestis* isolates leads to increased pathogen evasion of host innate immune defenses.

activity of RcsB, a DNA-binding protein involved in the negative regulation of biofilm synthesis genes. Loss of functional RcsA decreases the binding stability and repressor activity of RcsB, which is unable to fully repress biofilm synthesis independently.

In 2008, Sun and colleagues determined that expressing the functional *Y. pseudotuberculosis rcsA* gene in a wild type *Y. pestis* background not only abolished *in vitro* biofilm formation, but also significantly decreased flea blockage *in vivo* (522). In contrast, when the reciprocal experiment was performed, and the functional *rcsA* gene *in Y. pseudotuberculosis* was replaced with the *Y. pestis* pseudogene, the resulting strain was able to form biofilms in a *C. elegans* model, unlike its wild type parent. The presence of a functional RcsA inhibits biofilm formation, which would be incompatible with the *Y pestis* lifestyle in the flea. *rcsA*, therefore, is an AVG for *Y. pestis.*

The most common inactivating mutation of *rcsA* is a 30 base pair (bp) duplication insertion midway through the reading frame, verified not only in the main subspecies *pestis*, but also suspected to be prevalent in all other subspecies (*caucasica, altaica, hissarica and ulegeica*) (155). This result suggests that inactivation of *rcsA* likely occurred early in *Y. pestis* evolution, possibly the result of considerable selective pressure upon the bacteria to inactivate *rcsA* and allow for biofilm formation in order to enhance transmission from the flea. The only other known mutation of *rcsA* in *Y. pestis* occurs in the strain Antiqua, which lacks the characteristic 30 bp insertion but instead carries a 1.2 kb putative transposase gene inserted midway through the reading frame. Although functionality of this interrupted Antiqua *rcsA* has not yet been experimentally determined, it is probably inactive.

The loss of a biofilm degradation mechanism also appears to contribute to the ability of *Y. pestis* to form biofilms in the flea. *Y. pestis* extracellular matrix formation in the midgut of the flea is dependent on expression of the *hms* genes, which synthesize a poly-β-1,6-*N*-acetyl-D-glucosamine biofilm (154). *nghA* (formerly *chb*) encodes an active glycosyl hydrolase in *Y. pseudotuberculosis*. In one of the *Y. pestis* strains tested by Erickson and colleagues, no β-hexosaminidase activity was detected, suggesting that the *Y. pestis nghA* protein is nonfunctional. An 11 bp deletion early in the *Y. pestis nghA* gene leads to a premature stop codon, likely resulting in a truncated and nonfunctional protein product. This inactivating mutation appears to be conserved in all *Y. pestis* lineages, while *Y. pseudotuberculosis* strains retain full-length *nghA*.

When a functional *nghA* from *Y. pseudotuberculosis* is expressed in *Y. pestis*, the resulting product is able to cleave β-linked *N*-acetylglucosamine residues from the HMS-dependent extracellular matrix, ultimately destabilizing formed biofilms (154). Correspondingly, although this strain colonizes the midgut, similar to *Y. pseudotuberculosis*, biofilm formation on the flea proventriculus is markedly decreased. These experiments suggest that *Y. pestis* has adapted to its flea vector by selecting against the gene responsible for degradation of the extracellular matrix.

In addition to differences in the ability to form biofilms, *Y. pestis* and *Y. pseudotuberculosis* also exhibit another striking dissimilarity: *Y. pestis* lacks the ability to convert its LPS lipid A from tetra-acylated to hexa-acylated (365). Hexa-acylation, a post-translational modification of the lipid A subunit of bacterial LPS, is critical to provoking a potent immune response through interactions with CD14 and Toll-Like Receptor 4 (TLR4) on the host cell surface. Due to this deficit in lipid A hexa-acylation,

infection with *Y. pestis* induces only a weak innate immune response, and ultimately gives the bacteria a direct survival advantage *in vivo*. In contrast, *Y. pseudotuberculosis* displays hexa-acylated lipid A at the host temperature of 37° C, and produces an LPS capable of activating TLR4 (450).

Hexa-acylation of lipid A is carried out by two proteins: LpxL and LpxP. LpxL is a lauroyl acyltransferase that attaches secondary acyl chains to the tetra-acylated lipid A subunit (441). The *lpxL* gene is completely absent from all sequenced strains of *Y. pestis* (365). LpxP, an acyltransferase produced by *Y. pestis*, is active exclusively under cold shock conditions, and cannot rescue hexa-acylation in an *lpxL* mutant within normal host temperatures (441).

When Montminey and colleagues expressed a functional *lpxL* from *E. coli* in a wild type *Y. pestis* strain, the bacteria strongly activated human peripheral blood mononuclear cells compared to the wild type strain, as evidenced by an increase of TNF, IL-6, and IL-8 from these host cells (365). In a mouse model the wild type strain predictably resulted in 100% mortality, while the strain expressing *lpxL* did not result in any noticeable signs of disease. Mice infected with this latter strain had significantly lower spleen bacterial titers, in addition to increases in TNF levels and the appearance of liver microabscesses, indicative of a robust immune response. This host protection was TLR4-dependent, as TLR4-deficient mice infected with the strain expressing *lpxL* succumbed to infection. Conclusively, it was demonstrated that when the lipid A of *Y. pestis* undergoes hexa-acylation, the immune system is able to effectively recognize and mount an appropriate immune response against the infection. To optimize pathogenesis

and increase evasion of host immune responses, *Y. pestis* deleted *lpxL*, which joins *rcsA* and *nghA* as a *Y. pestis* AVG.

Montminey and colleagues proposed that this new AVG might be a potential vaccine candidate, and successfully tested this hypothesis in a mouse model of infection. Mice infected with the *Y. pestis* strain expressing *lpxL* were completely protected against challenge with wild type *Y. pestis* at least 40 days post-inoculation (365). This utilization of an AVG demonstrates the powerful potential for practical application of such studies, and further illustrates the significance of continuing to search for other AVGs in bacterial pathogens.

Francisella tularensis

The genus *Francisella*, composed of several facultative, intracellular zoonotic pathogens, is traditionally divided into two species: *F. philomiragia* and *F. tularensis* (526). *F. philomiragia* is a pathogen of muskrat and fish, though it may rarely cause disease in immunocompromised humans. Both virulence and transmission of this organism is poorly understood (250). *Francisella tularensis* is further split into four main subspecies: *F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*, *F. tularensis* subsp. *mediasiatica*, and *F. tularensis* subsp. *novicida*. For the sake of convenience, we will refer to these subspecies as *F. tularensis, F. holarctica, F. mediasiatica*, and *F. novicida.*

F. tularensis (also known as *F. tularensis* Type A), the most virulent subspecies of this subset, infects a broad mammalian host range and is most commonly spread via a wide variety of arthropod vectors, although less common routes of infection may include aerosolization or direct contact with infected animal meat (150). In humans, infection

manifests as a potentially fatal ulceroglandular or pneumonic tularemia. Strains of *F. holarctica* (also known as *F. tularensis* Type B) also infect healthy individuals, but are only moderately virulent compared to strains of *F. tularensis*. *F. mediasiatica* and *F. novicida*, in contrast, typically only present as human pathogens in immunocompromised individuals. Based on phylogenetic analysis, *F. novidica* likely branched first from an ancestral *Francisella* lineage, followed by *F. tularensis* and *F. mediasiatica*, which likely diverged at around the same time. *F. holarctica*, which branched last, is thought to be the most recent subspecies to emerge (526).

The *F. novicida* PepO is an M13 zinc metalloprotease that cleaves the neuropeptide metenkephalin *in vitro* (223). This secreted metalloprotease is an orthologue of the mammalian enzyme ECE-1, which cleaves pro-endothelin into the potent vasoconstrictor endothelin. Bacterial PepO proteins from other species, including *Streptococcus* and *Porphyromonas*, have been shown to mimic the activity of the host vasoconstrictor, and may in fact represent lateral gene transfer between eukaryotes and prokaryotes (32; 185). Strains of *F. tularensis*, *F. holarctica*, and *F. mediasiatica* have all lost the ability to secrete an active PepO (223).

pepO is secreted from *F. novicida* via a modified type four pilus (T4P), a system that in *Francisella* is postulated to act as a means of secreting proteins rather than as an actual pilus structure (223). When *pilC*, an inner membrane protein essential for T4P assembly, is deleted in *F. novicida*, the resulting mutant has significantly higher lethality in a mouse model of infection compared to its wild type parent. The bacterial burden in the spleen of mice infected with the mutant is correspondingly higher, leading to the hypothesis that bacterial spread from the initial site of infection is hindered. Due to the

predicted function of PepO, a *pepO* mutant was constructed and tested in the mouse model to determine if T4P-secreted PepO was responsible for inhibiting bacterial spread. Strikingly, like the *pilC* mutant, the *F. novicida pepO* deletion mutant displays enhanced mortality in the mouse model compared to the wild type parent, in addition to a higher bacterial load in the spleen (223). In a separate model of disease, in which mice are exposed to aerosolized wild type or *pepO* mutant strains, polymorphonuclear leukocyte (PMN) influx in the lung is increased in response to mutant but not wild type infection. These results suggest that increased vasoconstriction occurs in the presence of active PepO, as found in wild type strains of *F. novicida*, ultimately restricting both bacteria and PMN spread. In contrast, loss of a functional PepO in the more virulent *Francisella* subspecies likely allows for bacterial dissemination and increased pathogenicity.

Loss or inactivation of *pepO* in subspecies *F. tularensis* and *F. holarctica* stems from multiple mutation events. In *F. tularensis* strains, IS-mediated genomic rearrangements have replaced the N-terminal secretion signal, likely resulting in an inability of these strains to secrete PepO (223). In addition, there are numerous amino acid alterations of the *F. tularensis* PepO in comparison with the active PepO from *F. novicida*. The effect these changes have upon protein activity is unknown. *F. holarctica* strains carry a different IS-mediated genomic rearrangement that also targets the N-terminal secretion signal. In addition, these strains carry a nonsense mutation partway through the gene that truncates the protein and removes the protease domain, likely rendering the protein inactive (223). *F. mediasiatica* appears to have undergone the same genetic rearrangement as *F. holarctica*. The location of the nonsense mutation differs, however, suggesting that while early rearrangement to hamper secretion of this protein

was similar in an ancestral strain, actual inactivation of the gene may have been independent for each species. Finally, *pepO* is absent from the only sequenced *F. philomiragia* genome, suggesting that either this gene has been deleted from all or certain strains of this species, or *pepO* was never present in the ancestral progenitor strain.

The inactivation of *pepO* in the more lethal *Francisella* subspecies inhibits host vasoconstriction and permits the spread of the bacteria to systemic sites, allowing *pepO* to join the list of newly-described AVGs.

CONCLUDING REMARKS

Microbial evolution is the result of both positive and negative selection for specific traits that optimize the organism's fitness. In the case of pathogens, the acquisition of virulence traits must also correspond with the appropriate integration of those traits into the organism's current genome. This may ultimately result in inactivation of AVGs deleterious to the pathogen's new virulence factors. This fine-tuning process allows the pathogen to further adapt to its new host niche. It is likewise important to recognize that inactivation of AVGs can occur during the evolution of commensals to pathogens, such as *E. coli* to *Shigella*, or even during the evolution between pathogens, such as *Y. pseudotuberculosis* to *Y. pestis*.

In the last few years, several new AVGs have been described in the literature, increasing not only our repertoire of known AVGs, but also the number of pathogens that harbor these genes (Table 8). An impressively wide variety of different roles are attributed to AVGs in these organisms, and the current list of AVGs encompasses genes involved in metabolism, biofilm synthesis, LPS modification, lactose regulation, and protease activity, among others.

A more complete understanding of AVGs is vital to comprehending the evolution of pathogen virulence. More importantly, the study of antivirulence may assist in the uncovering of novel virulence targets, and contribute to the development of inhibitors of those targets. The first proof of principle application of an AVG, the successful utilization of an *lpxL*-expressing strain of *Y. pestis* as a potential vaccine candidate, is a promising example of the rewards of such research. Such work supports and highlights the importance of continuing the search for new AVGs.

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